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SANITIZER EFFICACY TOWARDS ATTACHED BACTERIA

BY

Tracy M. Mosteller

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

MASTER OF SCIENCE

in

Food Science and Technology

APPROVED:

J. Russell Bishop, Chairman

C.R. Hackney

M.D. Pierson

May, 1991
Blacksburg, Virginia

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Committee Chairman: J. Russell Bishop
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(ABSTRACT)

Pseudomonas fluorescens, Yersinia enterocolitica, and Listeria monocytogenes readily attach to both rubber and teflon surfaces. Once attached, a glycocalyx covering forms effectively protecting them from any sanitizer that passes over the surface. Therefore, sanitizers efficacy testing done in the laboratory with pure glycocalyx-free cultures could lead to false assumptions as to the sanitizer's true effectiveness under actual use conditions. Our objectives in this study were: (1) evaluate sanitizer efficacy of in-use concentrations toward bacteria attached to gasket materials, (2) examine attachment on rubber versus teflon gaskets, (3) examine different methods of enumeration, (4) compare kill of attached bacteria to suspension tests, (5) determine the minimum inhibitory concentrations of sanitizers. Iodophor, hypochlorite, acid anionic, peroxyacetic acid, fatty acid and QUAT sanitizers failed to provide an adequate log kill of bacteria attached in levels

of 10^4 to 10^5. Most of the tests showed that the log kill falls well short of a 3 log reduction goal. Plate counts, impedance microbiology, and the direct epifluorescent filter technique were tested as methods of enumeration. Impedance microbiology was the best method of enumeration, since it allows the estimation of both reversibly and irreversibly attached bacteria. Minimum inhibitory concentration tests demonstrated the increased resistance of attached bacteria as compared to cell suspensions.

ACKNOWLEDGEMENTS

Many thanks go to the faculty and staff of the Department of Food Science and Technology, especially to Rusty Bishop who has supported me throughout my master's program. I also extend my gratitude to Rob Byrne who was always there when I had a question or needed advice. I appreciate all the encouragement and help I received from my roommate Beth Sonneville. You've been a great friend these past two years and I hope we remain friends no matter where our lives may lead us. I must also thank the Evans' family, my "other" family, who have known me since "day 1". You've always been behind me in all my decisions. Most of all I'd like to thank my parents Charles and Carol Mosteller. This thesis is dedicated to them with love and pride. Someday I hope to repay them for all their love and support but right now a big "thank you" will have to suffice.

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INTRODUCTION

The food industry continues to face challenging and complex problems. Consumers are demanding high quality products with a longer shelf-life. One area of concentration for the food industry is cleaning and sanitizing. With a proper cleaning regime, processors can help ensure a safe and wholesome product. Currently, sanitizer evaluations are made using pure laboratory cultures. This could lead to expensive errors in the selection and evaluation of industrial biocides because attached bacteria are locked within a glycocalyx matrix.

Cleaning systems are implemented in food processing plants to produce a safe product with an acceptable level of quality. Many pathogenic and spoilage bacteria are capable of attachment to materials commonly used in food processing equipment such as stainless steel, and rubber and teflon gaskets. Attached bacteria may increase in number and detach on their own or by physical movement of product through a pipeline. This causes product quality concerns and a potential health hazard.

Although they appear to be smooth, gasket materials are covered with minute holes and cracks which provide an excellent harborage for bacteria. In addition, these pits give the adherent cells protection from antimicrobial agents and surfactants. Attached bacteria form a dynamic

microcolony environment. Growth and multiplication allow for the development of a biofilm layer. The biofilm layer contains many cells surrounded by a glycocalyx.

There is evidence to suggest that the glycocalyx of attached cells is common in the natural bacterial environment. Few cells exist in a free floating state. In pure laboratory cultures, the glycocalyx does not exist. There is no need to expend the energy needed to create and maintain a glycocalyx. Most often, the pure laboratory culture is used for sanitizer efficacy testing. This could lead to false assumptions as to the sanitizer's effectiveness. The glycocalyx covered colonies are more resistant to the sanitizers than pure cultures. A study of sanitizer efficacy toward attached bacteria would help determine the sanitizers true capabilities. Therefore, the objectives of this study were: (1) evaluate sanitizer efficacy of in-use concentrations toward bacteria attached to gasket materials, (2) examine attachment on rubber versus teflon gaskets, (3) examine different methods of enumeration, (4) compare kill of attached bacteria to suspension tests, (5) determine the minimum inhibitory concentrations of sanitizers.

LITERATURE REVIEW

BACTERIAL ATTACHMENT

Adherent microcolonies are the predominant form of bacterial growth in nature and many diseases. X-ray diffraction and studies of extracellular molecular interaction between carbohydrate chains indicate that bacteria in nature are surrounded by a thick, continuous, highly ordered hydrated polyanionic polysaccharide matrix (17). Bacteria can stick to surfaces ranging from the human tooth and intestines to a rock submerged in a rapidly moving stream (16). Bacteria have been recovered from surfaces in sea and seawater, from grains of sand and soil particles (74), root hairs of plants, and even the surfaces of other bacterial species (89). Primary attachment between bacteria and surfaces occurs reversibly through surface charges, van der Waals forces, and hydrophobic interactions (20, 46). This allows the development of proteinaceous or polysaccharide adhesions which form the irreversible secondary attachment. The cells become enveloped in a matrix of hydrated exopolysaccharides or glycocalyx (18, 88). Dunsmore (26) stated that an organism may attach to surfaces of equipment by: (1) direct attachment, (2) attachment to soil which is attached to a surface (3)

incorporation in a soil mass which is attached to a surface. The glycocalyx has been described as being any polysaccharide containing component outside the cell wall. Glycocalyces can be subdivided into two groups:

1. S layer - a regular array of glycoprotein subunits at the cell surface.

2. Capsules - a fibrous matrix at the cell surface with varying thickness; (a) rigid - structurally adherent capsule which can exclude certain molecules (i.e. India Ink, nigrosin); (b) flexible - similar to the rigid structure but does not exclude molecules; (c) integral - usually intimately associated with the cell surface; (d) peripheral - may not remain with the cell under all circumstances (17).

Until recently, bacterial attachment has not received much attention. This may be due to the cells lack of ability to produce a glycocalyx in pure culture. A bacterial cell must expend energy to generate and maintain a glycocalyx. In the protected environment of the pure laboratory culture, the glycocalyx is a luxury which provide no selective advantage (16, 17). While the bacteria are attached, the glycocalyx allows for a homeostatic state and minimizes environmental fluctuations (10). The glycocalyx matrix accomplishes this by concentrating nutrients and protecting embedded cells from surfactants, antimicrobial

agents, and phagocytic cells (88).

It has been postulated that bacterial attachment confers some antibiotic resistance to the cells (18, 46, 88). Gristina et al. (46) suggested that the glycocalyx acts as an ion exchange resin whose binding sites must be largely satisfied before charged molecules such as antibiotics can penetrate. In a study of disinfectant testing, Cole et al. (15) demonstrated that a thick mat of interlacing cells existed at least 10 um thick. This mat of cells significantly increased the number of positive cells in the use-dilution method. Notermans and Kampelmacher (78) showed that some mesophilic bacteria, such as E. coli, Klebsiella sp. and Salmonella oranienberg, are more heat-resistant when attached to poultry skins. Kotsides (59) found that attachment fibrils are not adversely affected by a sodium hypochlorite treatment. This finding is supported by Schwach (96) who stated that if the attachment fibrils are composed of mucopolysaccharide then it should not be surprising that sodium hypochlorite does not affect them. She goes on to suggest that additional research is needed to determine the effect of attachment during and after the cleaning and sanitizing of food contact surfaces. Brown and co-workers (10) warned against the assumption that resistance patterns are due to biofilm development. They

point out that nutrient deprivation and growth rate will contribute to antibiotic resistance of biofilm cells.

In addition to studies of resistance patterns, there have been several studies recently to determine where and when bacterial attachment will occur (1, 21, 22, 23, 65, 66, 71, 72, 73, 74, 101). Stanley (101) showed that when brought into contact with stainless steel, Pseudomonas aeruginosa cells begin to attach in < 1 minute and the number of cells increased with time. In the same study, it was determined that dead cells are capable of reduced but significant attachment. These nonviable cells provide a primary biofilm layer to which other viable cells might easily adhere. A similar conclusion was reached by Meadows (74) in an experiment to determine the attachment of bacteria to solid surfaces. According to the author, the adherence of nonliving cells proved that the attachment is of ecological advantage to the bacterium but solely reflects the effects of physicochemical forces. It was shown that the number of UV radiation-killed bacteria attaching were slightly reduced compared to the controls, while the number of heat and formalin killed cells that attached were lower. This demonstrated that the attachment depends on cell wall integrity. Absolm et al. (1) found that they could predict the number of bacteria adhering per unit surface area by the

use of thermodynamic equations. Maxcy and Shahani (72) demonstrated that product contaminants did not arise from a deposition/growth/recovery cycle but were the result of the presence of bacteria in harborages.

In an effort to examine environmental factors affecting survival of attached cells, McEldowney and Fletcher (73) studied the effects of temperature and relative humidity. They found that Gram negative and Gram positive survival was similar, and that survival was shortest at 25°C and longest at 4°C. Pseudomonas subspecies underwent cell division at 0% relative humidity. Water may have been conserved within the matrix or the highly hydrated glycocalyx could have provided enough moisture for growth and survival. This demonstrates the dynamic situation within the biomatrix - bacteria growing, dying, lysing, and resupplying nutrients to other cells continuously. Studies by Czechowski (21, 22, 23,) have shown that bacteria readily attach to food processing equipment. He has shown that Pseudomonas fluorescens, Salmonella typhimurium, E. coli, Yersinia enterocolitica, and Listeria monocytogenes all easily attach to surfaces used in food processing such as buna-N rubber and teflon gaskets, and stainless steel (21). By doing in vivo testing, it was demonstrated that gasket materials left in place for extended periods of time become heavily

contaminated and may serve as a potential harborage for bacteria (22). As cited in a review of attachment of bacteria to meat surfaces, Firstenberg-Eden (30) found that bacteria which contact poultry surfaces early in the process are extremely difficult to remove at the end of the process. Attachment allows cells present in low numbers in the rinse water to be concentrated at the meat surface. She goes on to point out that irreversibly attached bacteria may not be as hazardous to the consumer as the reversibly attached cells. The loosely attached bacteria are more likely to be spread throughout the kitchen causing cross-contamination. She also stated that firmly attached bacteria will not be counted by the conventional rinse and swab methods. Therefore, only a part of the total flora will be seen and it will result in low total numbers since some bacterial strains attach better than others.

The problem of bacterial attachment could lead to expensive errors in the selection and evaluation of industrial biocides. Standard laboratory practices to evaluate sanitizers are designed for reproducibility in any laboratory, but the results may lack relevance to actual in-use conditions (93). Sanitizer evaluations are made using pure laboratory cultures which produce no glycocalyx. A sanitizer which effectively destroys laboratory cultures may

fail to kill glycocalyx-enclosed microcolonies on surfaces which are to be contamination-free (17).

Herald and Zottola (49) pointed out that the presence of attached bacteria could cause potential health hazards and product quality concerns. A biofilm layer makes the cleaning and sanitation of the plant and equipment very significant. Bacterial contamination and colonization of milk contact surfaces are likely to be an important source of subsequent contamination of the product (61, 65). Dunsmore (26) stated that bacterial injury should not be overlooked. He showed that injured bacteria are capable of repair and limited toxin production. Some cells can be damaged by the cleaning and sanitizing system but when the stress is removed, these cells will undergo repair and may contaminate the product as it passes over the surface. A similar conclusion was reached by Stone and Zottola (103). They showed that if the cleaning procedure was inadequate then viable, attached unremoved cells remain. Kotsides (59, 60) revealed that some current sanitation practices will have to change in order to prevent post-pasteurization contamination of fluid milk by bacteria attached to gaskets. He advocated either more frequent replacement of gaskets or better sanitation methods.

ENUMERATION

Plate Counts

The conventional standard plate count (SPC) is probably the most widely accepted method for determining bacterial numbers. Recently, the 3M corporation introduced an alternative which is comparable to the traditional agar pour plate method and is known as Petrifilm (5, 12, 41, 77). Petrifilm is a dry film medium consisting of two layers. The base film contains dehydrated standard methods medium and the upper film is coated with a cold water soluble gelling agent and a tetrazolium indicator dye. Ginn et al. (41) proposed that Petrifilm AC is a suitable alternative to the SPC. They obtained good correlation (r=0.946) between the two methods and the repeatability was essentially the same. Bishop and Juan (5) obtained a good correlation of Petrifilm AC to the standard plate count (SPC) of raw milk with a correlation value of 0.971. Byrne et al. (12) used both petrifilm and traditional agar methods for bacterial enumeration. They found that either method could be used for estimating the quality of fluid milk products.

Plate count methods have received some criticism lately. Sharpe (97) stated that plate counts and other similar tests have no place in the future. Firstenberg-Eden

(32) pointed out the inherent limitations of plate count methods. First, the assumption that one colony arises from one bacterium is false because microorganisms naturally tend to exist in clumps or clusters. Second, cells must be able to form visible colonies under the test conditions in order to be enumerated. No plate count method can be expected to include all bacteria originally present in the sample. Finally, sampling errors can not be eliminated. Weighing samples, pipetting dilutions, and enumerating colonies contribute to inaccurate results.

Direct Epifluorescent Filter Technique

Assessment of microbial contamination in a short period of time is necessary to allow corrective action to be taken during production periods. The Direct Epifluorescent Filter Technique (DEFT) takes less than 30 minutes to complete and is a reliable method of estimating bacteria. Microorganisms can also be distinguished on the basis of morphology.

Acridine orange (AO) is a fluorochrome that absorbs UV light and emits visible light. It is the stain of choice with DEFT methodology. The AO cations bind to every third base pair of the double stranded DNA which prevents dye-dye

interaction. This produces the orthochromatic (green) fluorescence. When bound to the single stranded RNA, AO attaches to almost every nucleotide. Due to the flexibility of the coil, dye-dye interaction occurs which leads to metachromatic (orange-red) fluorescence (50, 83, 92). This phenomenon allows the distinction between viable and nonviable cells. Since nonviable cells have little or no RNA, they fluoresce green.

Pettipher, et al. (85) reported that the DEFT count had a correlation of 0.91 with plate counts. It has also been shown that the DEFT count can be used to enumerate as few as 6000 bacteria/ml in heat treated milk and milk products (86). Oppong (80) examined this method for dry foods and found it to be superior to conventional methods. A collaborative study conducted by Pettipher, et al. (84) revealed that each laboratory demonstrated a highly significant relationship between the DEFT and plate counts with an average correlation of 0.9, with no significant differences between operators. Kroll and Rodriques (62) have shown that the DEFT and plate count methods can give a good prediction of the keeping quality of pasteurized cream. The main advantage of the DEFT method is its time savings. Hunter and McCorquondale (54) examined the hygienic conditions of milking equipment. They found the DEFT method

useful because results could be obtained on the same day as the farm visit. DEFT has also been used for testing other food products such as meat and fish with a correlation of 0.91 to plate counts (87).

DEFT is a rapid enumeration technique which takes < 30 minutes to complete. It correlates well to plate counts and can be used when results are needed quickly.

Impedance Microbiology

As bacteria grow they change the chemical composition of the growth media. Metabolic activity results in the conversion of nonelectrolytes to ionically charged species (i.e. pyruvate -> pyruvic acid) (35). This knowledge is the basis for impedance microbiology, a rapid automated method which can be used to estimate the concentration of organisms initially present in a sample. Cady, et al. (13) stated that these chemical changes alter the electrical resistance or impedance. A sensitive monitor will detect the impedance changes caused by the increasing microbial population. The impedance in a growth medium remains relatively constant until the microbial population reaches a threshold level of approximately 10^6 to 10^7 cells/ml. Firstenberg-Eden (32)

described the time required for the initial inoculum to reach the threshold level as the impedance detection time (IDT). When an IDT occurs, it is automatically registered by the Bactometer Processing Unit (BPU). A sample with a higher initial level of bacteria will reach a detection time quicker and earlier than those with lower numbers of cells. As early as 1926, Parson and Sturges (81) noticed a direct proportionality between conductivity and ammonia production in the case of putrefactive anaerobes. Allison (2) proposed that changes in conductivity correlated with enzymatic hydrolysis of proteins.

Impedance microbiology is a rapid, reliable and easy method of enumeration. It has been used to estimate coliforms in meat samples with a correlation coefficient of 0.90 between IDT and violet red bile agar (VRBA) counts (33). Impedance has also been used to examine raw milk samples (31, 34, 43). Firstenberg-Eden and Tricarico (34) showed correlations of -0.96, -0.95, and 0.96 between IDT and standard plate counts (SPC) for total, mesophilic, and psychrotrophic counts in raw milk, respectively. When compared to the SPC, impedance detection produced less variability among laboratories. In addition, the reproducibilty variance of the IDT was lower than that of the SPC (31).

Attempts have been made to accurately determine the potential shelf-life of dairy products with the use of impedance (6, 7, 56, 70). Bishop, et al. (7) reported that IDT at 21°C and IDT at 18°C seemed to have a good correlation to shelf-life with correlation coefficients of 0.88 and 0.87, respectively. The use of impedance has definite advantages over the Moseley Keeping Quality Test. The Moseley test takes 7-9 days to complete, but impedance microbiology can provide results within 48 hours. Similar results were reported by Martins, et al. (70). In addition to these uses, impedance microbiology has been used for the detection of post-pasteurization contamination of cream and for niacin assays (29, 45).

Impedance microbiology is a rapid and reliable method with a variety of applications. This method is attractive to the dairy industry since results can be obtained quickly. This allows sanitation, shipping, and reprocessing decisions to be made before serious problems develop.

CLEANING AND SANITIZING

It is essential that all product contact surfaces remain clean in order to prevent any contamination of the

final food product. Dunsmore, et al. (26) defined a "cleaning system" as those practices which maintain the product-contact surfaces in a condition that ensures that they do not impair the quality of the final food product. A cleaning system is based on three parameters. It must remove any food residues which can serve as a source of nutrients for microorganisms that remain on the surface. It must also destroy any cells which were not physically removed with the food residues. Equipment must be stored in conditions that discourage the growth of surviving organisms during the intercycle period.

Cleaning and sanitizing are two separate components of the whole cleaning system. Cleaning is defined as the irreversible removal of soil. Sanitizing is a process that reduces the microbial load to an acceptable level consistent with quality control and hygienic standards. These two components are complimentary processes; neither can effectively achieve the desired results alone. Dunsmore (25) suggested that the detergent is the most important system component for controlling bacterial numbers, with the sanitizer exhibiting some control and the rinses very little. Forwalter (36) stated that effective cleaning of equipment is 90% of the overall sanitizing job. Although cleaning reduces bacterial numbers on surfaces, it

cannot eliminate all types of bacteria. Sanitation will eliminate nearly all of the remaining viable microorganisms. Pathogenic and spoilage bacteria must be removed or destroyed to insure the safety and quality of the product.

Four factors determine the effectiveness of chemical cleaners. Increasing the concentration can increase effectiveness, however, this is probably the least important factor. An increased temperature decreases the bonds between soil and surface. This increases the solubility of the soil and speeds chemical reactions. If all other factors are constant, cleaning can be increased by an increased contact time. In addition, external mechanical energy must be applied for effective cleaning. Chemical cleaners are designed to modify the nature of water so that it can penetrate, dislodge, and suspend soil particles. Water itself can be a good cleaning agent if enough external energy is applied. Cleaners act to decrease the external energy requirements by increasing the internal energy potential of water. Harper (47) described cleaning in terms of a series of five basic steps. First, prerinsing serves to reduce the soil load. Next, intimate contact of detergent solution and soil is accomplished by good wetting and penetrating properties. Soil is then displaced by saponifying the fat, peptizing the proteins, and dissolving

the minerals. Dispersion, defloculation, and emulsification suspend the soil in the solvent. Finally, redeposition is prevented by providing good rinsing properties. The main methods by which bacteria are controlled are physical removal or inactivation by sanitizers. Like cleaners, sanitizers are affected by concentration, temperature, and time of exposure. In addition, pH, cleanliness of the equipment, and water hardness can determine the effectiveness of sanitizers. Sanitizer performance depends on the substrate, soil, formulation, application method of the sanitizer, and the cleaning system in which it is applied (26). When examining sanitizer efficacy, it is important to remember that the number of surviving organisms, and not the number killed, is the significant factor (3).

SANITIZERS

Chlorine

In 1881, Koch demonstrated in the laboratory, under controlled conditions, the lethal effects of hypochlorites on pure cultures. The first large scale use of chlorine came in 1908 with the purification of water in Chicago. Since that time, chlorine has been used for a variety of

purposes; such as treatment of drinking water, waste water treatment, sanitation of equipment and surfaces, and treatment of some food products (53). Chlorine related compounds can be subdivided into five categories: (1) liquid chlorine, (2) hypochlorites, (3) inorganic chloramines, (4) organic chloramines, (5) chlorine dioxide. The hypochlorites (HOCl) appear to be the most active form of chlorine. This is probably the oldest and most widely used form: they are (1) proven and powerful germicides that can destroy a broad spectrum of microorganisms, (2) deodorizers, (3) nonpoisonous to humans at in-use concentrations, (4) free of poisonous residues, (5) colorless and nonstaining, (6) easy to handle and (7) economical (27). Hypochlorites are not effective in all circumstances. They have a short shelf-life, a penetrating and irritating odor, corrosiveness on some metals, and are adversely affected by organic matter (27, 40, 104).

Several theories have been proposed to explain the method of inactivation. It was originally thought that the action of chlorine was due to the formation of toxic N-chloro compounds, inhibition of glucose oxidation, oxidation of sulfhydryl groups (38, 39, 69). Other experiments have demonstrated that chlorine may interfere with the transportation of extracellular nutrients by impairing

membrane functions. Chlorine has also been shown to be effective against spores (63, 106). Kulikovsky, et al. (63) found that spores exposed to chlorine lose Ca++, DPA, RNA, and DNA due to altered permeability.

When added to an aqueous solution, hypochlorites will form hypochlorous acid which will dissociate to produce hydrogen and hypochlorite ions:

$$NaOCl + H_2O \rightarrow NaOH + HOCl$$

$$HOCl \rightarrow H+ + OCl-$$

The dissociation of hypochlorous acid to hypochlorite ion is greatly influenced by such factors as pH, temperature, and ionic strength (75). A pH range of 6-8 provides the environment for effective biocidal action. At pH 4 and below, the solution decomposes and liberates chlorine gas.

Iodophor

Like chlorine, iodine has been useful in water treatment and sanitation of processing equipment and surfaces. Elemental iodine is highly reactive and is thought to be the active form of iodine sanitizers (79):

pH

ACID ALKALINE

I_2	->	HOI	->	OI-	->	IO_3
Greatest Activity		Some Activity		Inactive Species		Inactive Species

Iodine is destructive toward a complete spectrum of microorganisms. Compared to vegetative cells, spores are ten to a thousand times more resistant. Iodine and chlorine are equally effective against vegetative cells (44, 48, 68, 76). Iodine appears to be less effective in inactivating spores (19). Its lethal action is rapid and acquired resistance to iodine does not occur. Except for low concentrations, effectiveness is not significantly reduced in the presence of organic matter. By itself, iodine is an effective sanitizer but has some serious disadvantages. Iodine is toxic, corrosive to metal and only slightly soluble in aqueous solutions. It can be a harsh irritant to the skin and mucous membranes. Aqueous solutions and tinctures evaporate quickly leaving iodine crystal on the epithelial cells causing irritation (14).

In 1949, Shelanski (104) found that certain surface-active agents such as polyvinylpyrrolidone could solubilize iodine to form a complex which has the germicidal properties of iodine but without the undesirable properties. He called

these complexes iodophores, from the Latin iodum meaning iodine and the Greek phorein meaning to carry. Iodophors are organic complexes with iodine trapped in the micelles of a surface-active agent. This increases stability and solubility. When diluted with water, the micelles disperse which liberates the iodine (102).

The exact destructive mechanism of iodine has not been fully elucidated but it is assumed that it interferes with protein function (42). Iodine reacts with basic N-H bonds that make up some amino acids and bases of nucleotides. This prevents hydrogen bonding and causes a loss of protein function. Iodine may also oxidize the S-H group of cysteine causing the disruption of disulfide bridges which can be an important factor in protein synthesis. Iodine reacts with the phenolic group of tyrosine which causes a steric hinderance in H-bonding of the phenolic OH group.

Although chlorine sanitizers are more widely used, iodophors are useful for disinfection of equipment, utensils, and work surfaces. A strongly acid iodophor can be used to prevent and remove milkstone formations from dairy equipment (40). This is possible because the optimum pH for use is 3-4. Iodine gives milkstone a yellow tint which aids in cleaning visualization. The surfactants that accompany iodine in the iodophor complex help to remove some

soils left on equipment surfaces.

Acid Anionic

Acid anionic sanitizers are a combination of organic or inorganic acid with a surfactant, such as sulfonated oleic acid. The addition of surface-active agents lowers the surface tension on bacterial membranes (9). Because it is acidic in nature, this sanitizer has the added advantage of removing mineral deposits that cause milkstone, eggstone, and beerstone. The low pH and surfactant activity provide additional germicidal effects.

Acid anionic sanitizers are classified in the general category of ionic surfactant type. These compounds are composed of two principle functional groups, a lipophilic group and a hydrophilic group. Upon dilution, ionization occurs, and the two groups produce a net charge for the molecule. Above a pH of 6.0, acid anionic sanitizers show only marginal bactericidal activity. Below pH 4.0, the negatively charged molecules react with the negatively charged bacterial surfaces. At a pH below 3.0, bacteria have a maximum positive surface charge and anionic sanitizers have maximum bactericidal activity (24).

Acid anionic sanitizers are thought to destroy bacteria by the general denaturation of cellular proteins. This

disrupts cell membranes and permeability barriers. In addition, enzymes essential to cell metabolism are inactivated (28). Acid anionic compounds are effective against a wide range of microorganisms, including thermodurics, bacteriophages, and most yeast strains (36, 67). Other advantages include the lack of an objectionable odor and production of a residual anti-bacterial film. It is effective in the presence of organic matter and in hard water. These sanitizers must be used at an acid pH which can make it corrosive to some metals. Acid anionic sanitizers are not effective in the destruction of most spores.

Peroxyacetic Acid

Peroxyacetic acid (also known as peracetic acid) has gained worldwide acceptance in the food industry for use in dairies, breweries, and meat and poultry processing plants. In comparison to hydrogen peroxide, this sanitizer is much more effective against a broad range of microorganisms at lower temperatures and concentrations (4, 95, 105). In 1902, Freer and Novy (37) first reported the effectiveness of peroxyacetic acid and in the mid-1960's, Spoessing and Muecke (98, 99, 100) made similar observations, but because

of stability and handling problems, acceptability was low.

A stabilized equilibrium mixture of hydrogen peroxide, peroxyacetic acid, acetic acid, and a stabilizer has allowed for widespread acceptance by eliminating many of the objections. The acetic acid reacts with hydrogen peroxide to form peroxyacetic acid + water.

Traces of peroxyacetic acid that remain on surfaces or enter the product are unstable and nontoxic. These residues quickly break down into oxygen, water, and acetic acid. Experiments to monitor decomposition of the residue used 65, 130, 250, and 500 ppm of the sanitizer in fresh milk. After 6 minutes, 65 ppm could no longer be detected (91). The same study indicated that $\leq$ 50 ppm of the sanitizer did not cause turbidity in beer, and did not adversely affect foam height, color, taste, odor, or pH.

Studies by the Danish Government Research Institute determined for the dairy industry that up to 10 ppm peroxyacetic acid did not adversely affect the color, pH, odor, and taste of the milk. Peroxyacetic acid is less corrosive than other sanitizers such as iodophor, acid anionic, and chlorine. It is also safe for use on most plastic and rubber compounds.

Peroxyacetic acid is universally effective against Gram positive and Gram negative bacteria, spore-forming bacteria,

viruses, and fungi (4, 8, 52, 55, 58, 91). More recently conducted tests indicate that it is effective against L. monocytogenes strains and bacteriophages (64, 94).

Fatty Acid Sanitizer

Fatty acid sanitizers contain octanoic and decanoic acids. Like acid anionic sanitizers, this sanitizer is effective against a broad range of microorganisms. Continual use is also effective in the prevention of mineralstone formation on processing equipment. Studies indicate that this sanitizer is effective against Salmonella and Listeria (57). Fatty acid sanitizers are effective on food contact surfaces as well as noncontact surfaces. It is noncorrosive to stainless steel equipment and can be used during an acid rinse cycle which lessens effluent discharge during clean-up. This sanitizer has the disadvantage of a fatty acid odor, but it is effective at a low pH and has low foaming properties.

Quaternary Ammonium Compounds

The quaternary ammonium compounds or QUATs are surface

active disinfectants with both hydrophilic and hydrophobic groups. For this reason, they are commonly referred to as "cationic detergents", although the detergent action is diminished at the concentration used for disinfection (14, 40). The surface-active positively charged nature of QUATs enhance the uptake of the molecules by the microorganisms.

Molecules tend to migrate forming a monomolecular layer at surfaces and interfaces, which gives the QUATs greater stability and a marked bacteriostatic property. This means that few free molecules will be found in solution. However, it also means that the QUATs cannot be easily rinsed off of surfaces.

QUATs are organically substituted ammonium compounds. Chemically, they have a nitrogen atom with a valency of 5 and four carbon atoms are directly linked to the nitrogen atom by covalent bonds. The general formula for QUATs is:

$$\begin{array}{ccc} & R_1 & \\ R_2 & N & X- \\ R_3 & R_4 & \end{array}$$

where the X- is usually a halogen and R_1, R_2, R_3, and R_4 are alkyl groups. At least one of the R groups is a long hydrocarbon chain derived from fatty acids with 8-18 carbon

atoms. The hydrophobic chain allows the molecules to congregate at interfaces. The nitrogen atom plus the four alkyl groups form the positively charged cation, which serves as the functional portion of the molecule, while the anion portion exhibits no antimicrobial activity (14, 40, 79). The cation has a high affinity for water and prevents separation at the aqueous phase (40). The classification of antimicrobial QUATs depends upon the nature of the R groups, the anion, and the number of quaternary nitrogen atoms.

At commonly used concentrations, QUATs are virtually colorless, odorless, tasteless, and noncorrosive, although cement, synthetic rubber, and aluminum may be adversely affected (82). They are only slightly toxic or irritating to human tissues. QUATs are able to withstand high temperatures and radiation sterilization. The poor rinsibility of QUATs can lead to undesirable traces in foods and throughout a processing plant.

QUATs are not equally effective against all microorganisms. They are very active against Gram positive cocci but are unreliable for killing Gram negative bacteria. The bactericidal concentration for S. aureus and fungi is ~ 50 ug/ml, but there is no sporicidal activity and little effectiveness against viruses although the lipophilic viruses are susceptible to some QUATs.

QUATs are most effective at a pH around 10. pH values in the neutral range are unfavorable. Similar to other antimicrobials, their activity is enhanced by increasing the temperature of the solution. The bactericidal properties are lowered in the presence of organic matter especially blood, serum, and milk which inactivate QUATs by adsorption. Anionic soaps and synthetic detergents which carry the opposite electrical charge may also inactivate QUATs.

MATERIALS AND METHODS

Pseudomonas fluorescens ATCC 13525 was grown on Nutrient Agar (NA) (BBL Microbiology Systems, Cockeysville, MD) slants at 21°C. Yersinia enterocolitica ATCC 9610 and Listeria monocytogenes Strain Scott A were grown on Trypticase-soy Agar + 0.6% yeast extract (TSYA) (BBL Microbiology Systems, Cockeysville, MD) slants at 21°C and 32°C, respectively. Transfers were made on a monthly basis. 99 ml of two percent milk was steamed for five minutes at 121°C and stored at 2°C until use. The inoculating organism was subcultured into nutrient broth (NB) for Pseudomonas and T-soy + 0.6% yeast extract broth (TSYB) for Yersinia and Listeria on three consecutive days to ensure an actively growing culture.

Sanitizer Efficacy Test

Twenty ml of sterile agar was placed into a sterile French bottle. The agars used for growth were those previously mentioned. The bottle was placed horizontally for solidification of the agar. The test organism was removed from a slant with 5 ml of phosphate buffer and sterile 4 mm glass beads. The suspension was poured back

into the 99 ml dilution blank. Additional glass beads were added to the diluent which was shaken vigorously to break up clumps of bacteria. Two ml of this suspension was pipetted into prepared French bottle flats. After 15 to 20 minutes, the excess liquid was poured off and the flats were incubated at 21°C for 48 hours. Two ml of phosphate buffer and glass beads were added to each flat to "strip" the inoculum. This suspension was poured into a buchner funnel with Whatman No. 2 paper. The colonies were broken up by vacuum filtration. The Spectronic 21 (Milton Roy Co., WA) was used at 540 nm to standardize the culture to 10^{10} cfu/ml.

Prior to testing all disinfectant solutions, inoculum, and inactivation liquid were equilibrated to the test temperature of 25 $\pm$ 1°C. One ml of each test organism was added to a 250 ml Erlenmeyer flask containing 100 ml of sanitizer. After 30 seconds, one ml was removed and placed in 9 ml neutralizer blanks for 5 $\pm$ 1 min. Following the inactivation period, pour plates were done to determine the number of surviving organisms (51).

The following sanitizers were used: iodophor, chlorine, acid anionic (Diversey Wyandotte, Wyandotte, MI), peroxyacetic acid, quaternary ammonium compound, and fatty acid (Klenzade, Division of Ecolab, Inc., St. Paul, MN).

Neutralizer blanks were prepared according to AOAC procedures (51) with 10.4 ml of a 0.25 M buffer stock solution, 46.0 ml of AOAC Neutralizer stock, and 744 ml of deionized water. This mixture was dispensed 9.0 ml per test tube and sterilized at 121°C for 15 minutes. The buffer stock solution contained 34.0 ml of KH_2PO_4 dissolved in 500 ml of distilled water and diluted to 1 liter with the pH adjusted to 7.2. A neutralizer stock solution contained 40 g azolectin, 280 ml polysorbate 80, and 1.25 ml phosphate buffer. This solution was diluted to 1 liter with a pH of 7.2 and autoclaved at 121°C for 20 minutes.

Efficacy Towards Attached Bacteria

Buna-N rubber and teflon gasket materials were obtained from M.G. Newell, Greensboro, NC. Rubber gasket materials had an internal diameter of 40 mm and an external diameter of 60 mm with a thickness of 3.2 mm. Teflon gaskets were 2.2 mm thick with an internal diameter of 20 mm and an external diameter of 50 mm. Gasket materials were cut into pieces with a press using a 3.7 mm bore. Gasket surface area was calculated. The pieces were then rinsed with water, wrapped in aluminum foil and sterilized for 15 minutes at 121°C.

Milk blanks were warmed to room temperature and

inoculated with 1 ml of each of the test organisms. After a 4 hour incubation at 21°C, gasket pieces were added to the flask and exposed to the bacteria for 12 hours at 21°C. To better simulate movement of product through a pipeline, a Controlled Environment Incubator Shaker (New Brunswick, NJ) was set at 120 rpm.

Six sanitizers were used in the following parts per million (ppm) concentrations of the active ingredient: iodophor, 25; chlorine, 200; acid anionic, 200; peroxyacetic acid, 200; fatty acid, 200; and QUAT, 200. Gasket pieces were removed from the inoculating milk, rinsed with phosphate buffer to remove loosely adhering cells, and exposed to 10 ml of sanitizer for 30 seconds. After exposure, the gaskets were placed in nine ml neutralizer blanks for 5 $\pm$ 1 minutes. To recover injured bacteria, some of the treated gaskets were placed in five ml nutrient broth and incubated at 21°C for two hours. Counts were obtained for initial attachment, survivors after sanitizer treatment, and injured cells. This study was replicated three times.

Impedance Microbiology

Impedance measurements were performed using a Bactometer Microbial Monitoring System Model - 120 (Vitek

Systems, St Louis, MO). Each module well was overlayed with 0.5 ml of modified plate count agar (MPCA) which contained the following ingredients in grams per liter: yeast extract, 20; tryptone, 20; dextrose, 4; and agar, 10. This agar layer prevented contact between gasket pieces and electrodes. Each well is filled with 1.5 ml of modified plate count broth (MPCB). Contaminated gasket pieces were placed in duplicate wells and impedance detection times (IDT) were automatically registered by the Bactometer Processing Unit (BPU). The IDTs for duplicate wells were averaged and each test was done in triplicate. In order to relate IDT with cell numbers, a standard curve was prepared. A series of dilutions were made using each test organism. These dilutions were monitored for IDT and plated on Petrifilm AC to determine cell number. Linear regression was used to analyze the data (SAS, SAS Institute, Inc. Cary, NC).

Direct Epifluorescent Filter Technique

The direct epifluorescent filter technique (DEFT) was used to enumerate reversibly and irreversibly attached cells. Gasket sections were vortexed with five ml phosphate buffer for one minute to remove the reversibly attached cells. Two ml of buffer was filtered through a nucleopore

polycarbonate membrane filter (0.6 um pore size, 25 mm diameter) mounted on a filter tower. The filters were overlayed with 1.0 ml of acridine orange (0.03% v/v) for 30 seconds, then filtered. Isopropyl alcohol was used to immediately destain the filters. Filters were removed, allowed to air dry, and mounted onto a microscope slide with nonfluorescent immersion oil. Another drop of immersion oil was added and a coverslip was placed on the filter.

Slides were counted using an Olympus BH-2 fluorescent microscope (Optical Elements Corporation, Washington, D.C.) with the 100X Plan D objective. Viable clumps of orange-fluorescing bacteria were counted as follows:

Ave. Number of Clumps/Field	Number of Fields Counted
0-10	15
11-25	10
26-50	6
51-75	3
76-100	2
>100	Dilute and Repeat

The average number of clumps per field was multiplied by a microscope factor (MF) to estimate the number of bacteria per ml (11). The microscope factor was calculated by the equation:

$$MF = \frac{\text{Area of membrane through which sample was filtered } (mm^2)}{\text{Microscope field area } (mm^2) \times \text{sample volume (ml)}}$$

Irreversibly attached cells were counted on the gasket

surface. After removal of the reversibly attached cells, gasket pieces were placed in a Petri dish and flooded with Kirkpatrick's fixative. Kirkpatrick's fixative is a 6:3:1 v/v mixture of isopropyl alcohol, chloroform, and formaldehyde. After three minutes, the samples were removed and rinsed with ethyl alcohol. A 0.3% solution of Sentol (Diversey Wyandotte, Wyandotte, MI) was added to another Petri dish and gasket sections were allowed to sit two minutes. The addition of Sentol effectively eliminates the green haze associated with milk deposits on the gasket surface after staining. Gaskets were then stained with 0.03% acridine orange for four minutes. Five fields were examined for orange-fluorescing clumps. The average number of clumps were multiplied by the gasket surface area to estimate the number of bacteria per mm^2 (21).

Plate Counts

One ml samples of the phosphate buffer used to remove reversibly attached bacteria in the DEFT method were used for plate counts. Pseudomonas fluorescens was enumerated using Petrifilm aerobic count (AC) at 21°C for 48 hours Yersinia enterocolitica and Listeria monocytogenes were enumerated using Petrifilm AC and TSYA. Plates were

incubated for 48 hours at 21°C for Yersinia and 32°C for Listeria (90).

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of cell suspensions was determined initially. One ml of the test culture was placed in 20 ml of sanitizer in a series of concentrations. Test tubes were examined for positive growth at 21°C. The lowest concentration which prevented growth was the MIC. This procedure was repeated using cells that were previously attached to gasket materials. A milk blank was inoculated with the test organisms and incubated 4 hours at 21°C. Gasket pieces were introduced and allowed to incubate 4 hours. At the end of this incubation period, gasket sections were removed and vortexed with 10 ml phosphate buffer. One ml of buffer was used to inoculate the 20 ml of sanitizer solution. Results were determined as previously described.

RESULTS AND DISCUSSION

Suspension tests are conducted to determine the efficacy of a sanitizer toward a particular organism. Each organism to be studied needs to be tested because bacteria react in specific and unique ways. Results of the suspension tests (Table 1) show that the sanitizers used in this study were quite effective in reducing the number of bacteria at least five log cycles or by 99.999%. The sanitizer efficacy test or Chamber's test requires that a sanitizer reduce a microbial population by five log or 99.999% after a 30 second exposure in order to be considered effective. Three different organisms were tested and all sanitizers at the in-use concentration produced an adequate reduction in cell numbers.

Standard curves were established to relate impedance detection times to cell number. Figures 1-3 represent the standard curves for Pseudomonas fluorescens, Yersinia enterocolitica, and Listeria monocytogenes, respectively. These curves plot the impedance detection times versus log number of cells over a range of 10^1 to 10^7. The impedance detection times can be converted to log cfu/ml with the linear regression equation (SAS, SAS Institute Inc., Cary, NC). Even though the suspension test resulted in a five log reduction, it cannot assumed that the sanitizer will

Table 1 - Number of surviving organisms from a suspension test.

Organism/Inoculum	IO	CL	PA	AA	FA	Q
Pseudomonas fluorescens 1.0×10^8	<1	<1	<1	<1	<1	<1
Listeria monocytogenes 1.4×10^8	<1	<1	<1	<1	<1	<1
Yersinia enterocolitica 4.4×10^8	<1	<1	<1	<1	<1	<1

IO = Iodophor CL = Hypochlorite PA = Peracetic Acid

AA = Acid Anionic FA = Fatty Acid Q = Quat n = 3

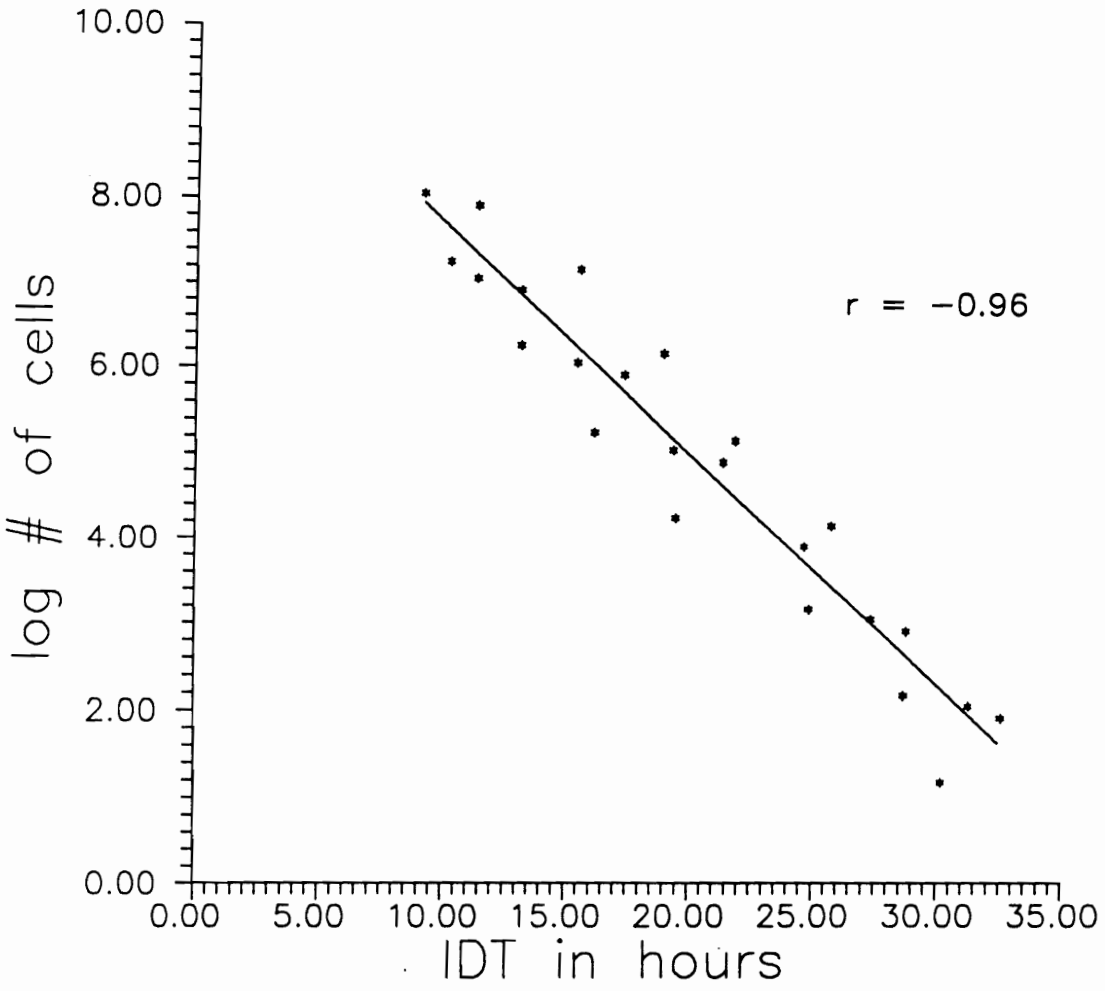

Figure 1 - Standard curve for <u>Pseudomonas fluorescens</u> enumeration by impedance detection. Log cfu/ml = -0.28 * TIME + 10.51. (n=24)

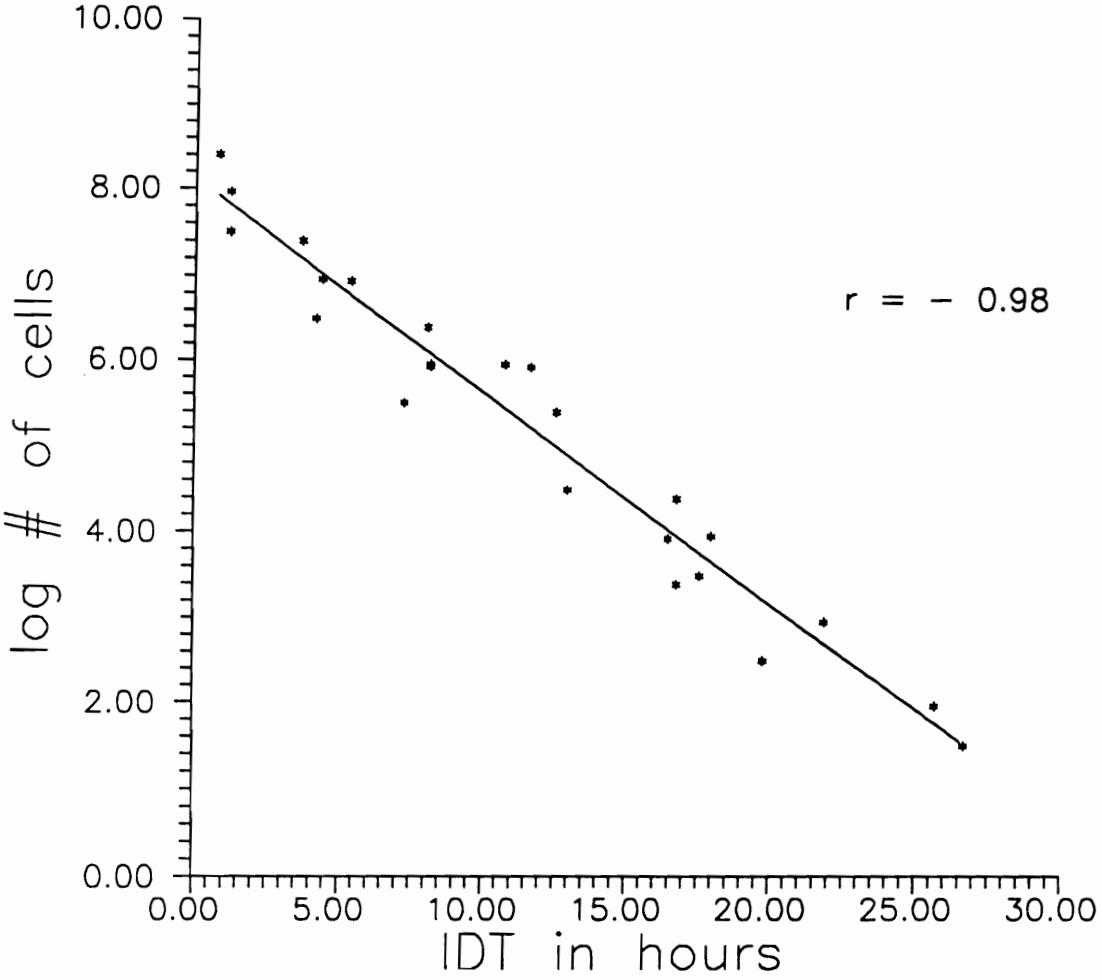

Figure 2 - Standard curve for <u>Yersinia enterocolitica</u> enumeration by impedance detection. Log cfu/ml = -0.25 * TIME + 8.11. (n=24)

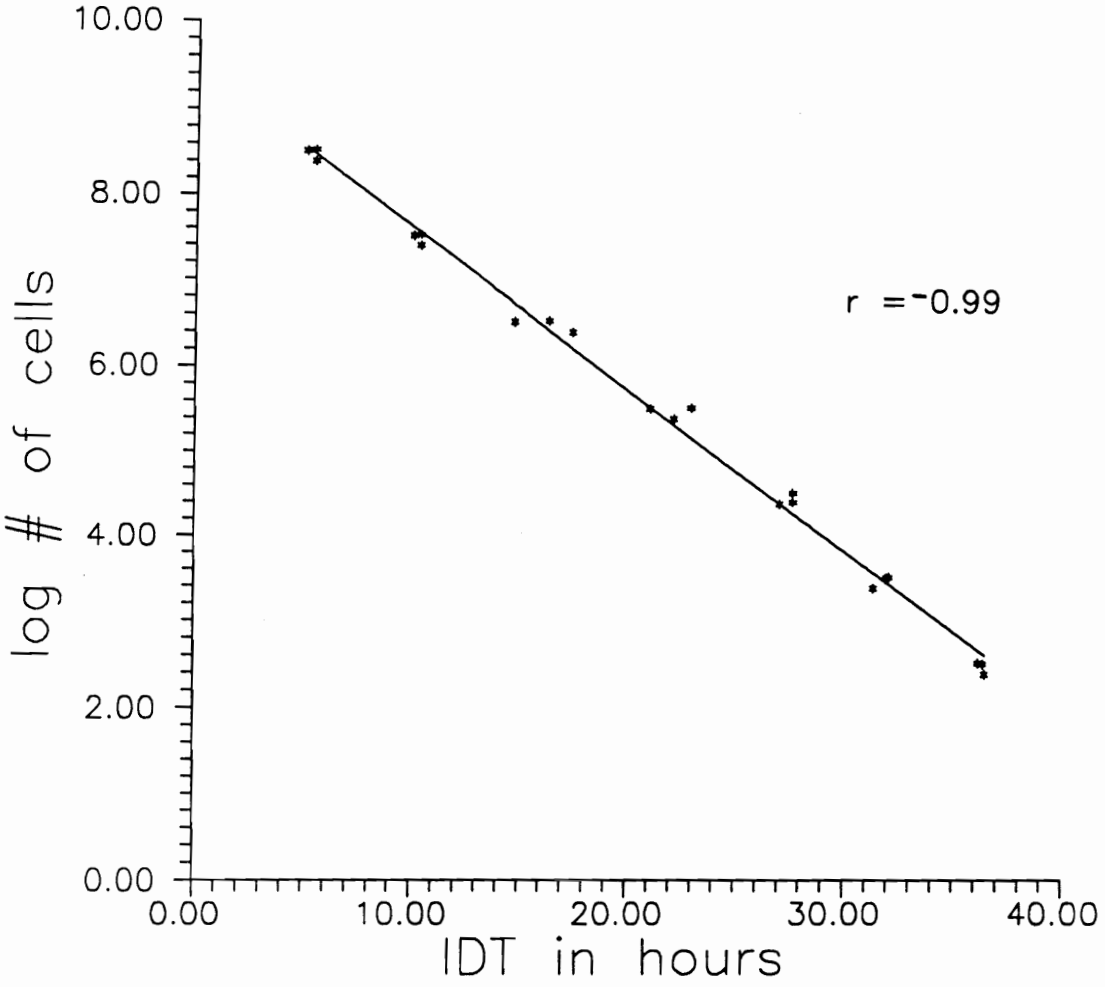

Figure 3 - Standard curve for Listeria monocytogenes enumeration by impedance detection. Log cfu/ml = -0.19 * TIME + 9.53. (n=21)

react with the same intensity toward attached bacteria. The goal reduction with attached bacteria was a three log or 99.9% reduction. This level was certainly attainable since bacteria will attach in the 10^4 to the 10^5 range. It appears that the three test organisms attached with greater frequency to the buna-N rubber substrate (Tables 2-7). This is not surprising given its tendency to be covered with pits and holes which can serve as primary attachment sites. Overall, Pseudomonas fluorescens attached to surfaces in the largest quantity. Bacteria that are attached are more resistant to sanitizers than those bacteria which exist in a free-floating state. Once attached, cells begin to bury themselves under a glycocalyx layer. Biomatrix, instead of biofilm, may be a better descriptive term for the cellular arrangement. The term biofilm connotates a uniform layer of cells when this is far from the truth. Microorganisms attach at favorable sites such as holes or imperfections in the surfaces.

In most cases, the iodophor sanitizer failed to reach the three log reduction goal with all of the test organisms (Table 2). The iodophor was effective in killing Pseudomonas and Yersinia attached to teflon. Although the bacteria readily attached to the surface, this clearly demonstrates the inability of cells to maintain tenacious adherence to the polymer surface.

Table 2 - Log kill after treatment with iodophor sanitizer

ENUMERATION METHOD	SURFACE		LOG OF BACTERIAL NUMBERS			
			INITIAL	TREATMENT*	RECOVERY**	LOG KILL
P	P.C.	R	5.47	4.43	3.70	1.04
S		T	4.75	2.52	2.40	2.23
E						
U	DEFT	R	5.12	4.82	4.60	0.30
D		T	4.80	4.02	4.37	0.78
O						
M	IDT	R	7.20	6.50	6.45	0.70
O		T	7.05	3.30	1.25	3.75
N						
A	DEFT-I	R	2.27	1.90	2.22	0.37
S		T	1.85	1.57	1.65	0.28
	P.C.	R	4.73	3.77	3.51	0.96
Y		T	4.54	1.45	1.40	3.09
E						
R	DEFT	R	4.10	3.60	3.68	0.50
S		T	3.89	3.56	3.49	0.33
I						
N	IDT	R	6.28	5.40	5.60	0.88
I		T	5.75	3.45	2.80	2.30
A						
	DEFT-I	R	1.35	0.66	1.18	0.69
		T	0.89	0.38	0.37	0.51
	P.C.	R	4.69	2.51	2.37	2.18
L		T	4.39	1.66	2.25	2.73
I						
S	DEFT	R	4.38	4.13	4.17	0.25
T		T	4.42	4.13	4.32	0.29
E						
R	IDT	R	6.30	5.42	5.42	0.88
I		T	6.42	3.80	4.50	2.62
A						
	DEFT-I	R	0.78	0.60	0.60	0.18
		T	1.19	0.60	0.68	0.59

P.C. = Plate count, IDT = Impedance Detection Time, DEFT = Direct Epifluorescent Filter Technique - Reversible, DEFT-I = Direct Epifluorecent Filter Technique - Irreversible, R = rubber, T = teflon, * = Exposure to 25 ppm iodophor for 30 seconds, ** = Recovery following a 2 h incubation in nutrient broth. n = 3.

Table 3 - Log kill after treatment with hypochlorite sanitizer

ENUMERATION METHOD	SURFACE		LOG OF BACTERIAL NUMBERS			
			INITIAL	TREATMENT*	RECOVERY**	LOG KILL
P	P.C.	R	5.39	2.23	2.40	3.16
S		T	4.58	1.42	1.73	3.16
E						
U	DEFT	R	5.25	4.80	4.57	0.45
D		T	4.74	4.36	4.40	0.38
O						
M	IDT	R	7.20	5.80	6.45	1.40
O		T	7.05	5.50	5.40	1.55
N						
A	DEFT-I	R	2.37	2.20	1.90	0.17
S		T	1.97	1.63	1.40	0.34
	P.C.	R	4.67	2.56	2.77	2.11
Y		T	4.29	1.10	1.78	3.19
E						
R	DEFT	R	4.05	2.91	3.64	1.14
S		T	3.92	2.24	3.47	1.68
I						
N	IDT	R	6.28	5.20	4.65	1.08
I		T	5.75	4.08	4.00	1.67
A						
	DEFT-I	R	1.81	0.64	0.79	1.17
		T	1.16	0.49	0.61	0.67
	P.C.	R	4.05	1.93	1.74	2.12
L		T	3.72	1.64	1.40	2.08
I						
S	DEFT	R	4.54	4.12	4.14	0.42
T		T	4.50	4.17	4.16	0.33
E						
R	IDT	R	6.30	4.60	4.90	1.70
I		T	6.42	5.15	4.65	1.27
A						
	DEFT-I	R	0.99	0.65	0.91	0.34
		T	0.78	0.42	0.50	0.36

P.C. = Plate count, IDT = Impedance Detection Time, DEFT = Direct Epifluorescent Filter Technique - Reversible, DEFT-I = Direct Epifluorecent Filter Technique - Irreversible, R = rubber, T = teflon, * = Exposure to 200 ppm hypochlorite for 30 seconds, ** = Recovery following a 2 h incubation in nutrient broth. n = 3.

Table 4 - Log kill after treatment with acid anionic sanitizer

ENUMERATION METHOD	SURFACE		LOG OF BACTERIAL NUMBERS			
			INITIAL	TREATMENT*	RECOVERY**	LOG KILL
PSEUDOMONAS	P.C.	R	5.25	4.27	3.97	0.98
		T	4.43	1.85	2.42	2.58
	DEFT	R	5.40	4.93	4.65	0.47
		T	4.83	4.30	4.42	0.53
	IDT	R	7.20	6.20	7.20	1.00
		T	7.05	5.10	4.42	1.95
	DEFT-I	R	2.30	1.80	2.10	0.50
		T	2.02	1.80	1.73	0.22
YERSINIA	P.C.	R	4.71	3.37	3.39	1.34
		T	4.05	1.66	1.30	2.39
	DEFT	R	4.13	3.64	2.90	0.64
		T	3.98	3.36	3.45	0.62
	IDT	R	6.28	5.30	5.50	0.98
		T	5.75	3.40	3.65	2.35
	DEFT-I	R	1.21	0.64	0.56	0.57
		T	1.01	0.84	0.79	0.17
LISTERIA	P.C.	R	<3.0	1.32	1.59	1.68
		T	<3.0	1.15	1.69	1.85
	DEFT	R	4.45	2.64	4.27	1.81
		T	4.56	3.98	4.23	0.58
	IDT	R	6.30	5.50	4.90	0.80
		T	6.42	5.65	5.40	0.77
	DEFT-I	R	1.10	0.71	0.87	0.39
		T	0.92	0.13	0.54	0.79

P.C. = Plate count, IDT = Impedance Detection Time, DEFT = Direct Epifluorescent Filter Technique - Reversible, DEFT-I = Direct Epifluorecent Filter Technique - Irreversible, R = rubber, T = teflon, * = Exposure to 200 ppm acid anionic sanitizer for 30 seconds, ** = Recovery following a 2 h incubation in nutrient broth. n = 3.

Table 5 - Log kill after treatment with peroxyacetic acid sanitizer

	ENUMERATION METHOD	SURFACE	LOG OF BACTERIAL NUMBERS			
			INITIAL	TREATMENT*	RECOVERY**	LOG KILL
P	P.C.	R	5.28	2.83	2.92	2.43
S		T	4.58	2.42	2.57	2.16
E						
U	DEFT	R	4.00	3.48	3.53	0.52
D		T	3.80	3.44	3.20	0.36
O						
M	IDT	R	7.20	6.05	6.00	1.15
O		T	7.05	4.40	4.80	2.65
N						
A	DEFT-I	R	1.78	1.14	1.16	0.64
S		T	1.81	1.26	1.50	0.55
	P.C.	R	4.99	3.98	4.00	1.01
Y		T	3.90	2.80	3.30	1.10
E						
R	DEFT	R	3.78	2.84	3.55	0.94
S		T	3.51	3.45	3.16	0.06
I						
N	IDT	R	6.28	5.20	4.70	1.08
I		T	5.75	2.85	2.90	2.90
A						
	DEFT-I	R	1.33	<1	0.25	1.33
		T	1.01	0.62	<1	0.39
	P.C.	R	5.05	3.90	4.33	1.15
L		T	3.84	3.22	3.73	0.62
I						
S	DEFT	R	3.97	3.08	3.00	0.89
T		T	3.69	3.25	3.00	0.44
E						
R	IDT	R	6.30	4.70	5.00	1.60
I		T	6.42	6.00	5.10	0.42
A						
	DEFT-I	R	1.04	0.30	0.40	0.74
		T	0.81	0.39	0.27	0.42

P.C. = Plate count, IDT = Impedance Detection Time, DEFT = Direct Epifluorescent Filter Technique - Reversible, DEFT-I = Direct Epifluorecent Filter Technique - Irreversible, R = rubber, T = teflon, * = Exposure to 200 ppm peroxyacetic sanitizer for 30 seconds, ** = Recovery following a 2 h incubation in nutrient broth. n = 3.

Table 6 - Log kill after treatment with fatty acid sanitizer

	ENUMERATION METHOD	SURFACE	LOG OF BACTERIAL NUMBERS			
			INITIAL	TREATMENT*	RECOVERY**	LOG KILL
P	P.C.	R	5.95	3.98	3.95	1.97
S		T	5.42	2.67	----	2.75
E						
U	DEFT	R	5.28	4.86	4.43	0.42
D		T	4.32	3.94	4.25	0.38
O						
M	IDT	R	7.20	6.20	6.85	1.00
O		T	7.05	3.25	3.61	3.80
N						
A	DEFT-I	R	2.25	2.05	1.77	0.20
S		T	1.72	1.35	1.50	0.37
	P.C.	R	4.88	3.33	3.45	1.55
Y		T	4.20	0.89	0.89	3.31
E						
R	DEFT	R	3.92	3.42	3.52	0.50
S		T	3.76	2.15	1.75	1.61
I						
N	IDT	R	6.28	5.45	5.75	0.83
I		T	5.75	3.00	3.10	2.75
A						
	DEFT-I	R	1.43	0.83	0.79	0.60
		T	1.01	0.64	0.81	0.37
	P.C.	R	4.08	2.64	2.46	1.44
L		T	3.74	1.47	2.49	2.27
I						
S	DEFT	R	4.20	3.93	4.06	0.27
T		T	4.11	3.75	3.94	0.36
E						
R	IDT	R	6.30	5.60	5.22	0.70
I		T	6.42	6.10	5.40	0.32
A						
	DEFT-I	R	1.20	0.53	0.72	0.67
		T	1.49	0.63	0.49	0.86

P.C. = Plate count, IDT = Impedance Detection Time, DEFT = Direct Epifluorescent Filter Technique - Reversible, DEFT-I = Direct Epifluorecent Filter Technique - Irreversible, R = rubber, T = teflon, * = Exposure to 200 ppm fatty acid sanitizer for 30 seconds, ** = Recovery following a 2 h incubation in nutrient broth. n = 3.

Table 7 - Log kill after treatment with QUAT sanitizer

ENUMERATION METHOD		SURFACE	LOG OF BACTERIAL NUMBERS			
			INITIAL	TREATMENT*	RECOVERY**	LOG KILL
P	P.C.	R	5.05	2.43	3.57	2.62
S		T	3.98	3.10	2.92	0.88
E						
U	DEFT	R	3.72	3.45	3.33	0.27
D		T	3.47	3.41	3.05	0.06
O						
M	IDT	R	7.20	6.85	7.17	0.35
O		T	7.05	5.18	5.85	1.87
N						
A	DEFT-I	R	1.61	0.50	0.56	1.11
S		T	1.53	0.94	0.66	0.59
	P.C.	R	5.33	4.18	4.40	1.15
Y		T	4.70	3.20	3.91	1.50
E						
R	DEFT	R	3.87	2.90	2.60	0.97
S		T	3.90	3.11	2.86	0.79
I						
N	IDT	R	6.28	5.40	5.00	0.88
I		T	5.75	4.60	4.40	1.15
A						
	DEFT-I	R	1.29	1.08	0.54	0.21
		T	1.36	0.52	0.77	0.84
	P.C.	R	4.89	3.94	4.29	0.95
L		T	3.95	2.79	3.26	1.16
I						
S	DEFT	R	3.87	2.74	3.10	1.13
T		T	3.29	3.20	3.10	0.09
E						
R	IDT	R	6.30	5.60	4.85	0.70
I		T	6.42	5.60	4.60	0.82
A						
	DEFT-I	R	0.88	0.42	0.25	0.46
		T	0.68	0.14	0.06	0.54

P.C. = Plate count, IDT = Impedance Detection Time, DEFT = Direct Epifluorescent Filter Technique - Reversible, DEFT-I = Direct Epifluorecent Filter Technique - Irreversible, R = rubber, T = teflon, * = Exposure to 200 ppm QUAT sanitizer for 30 seconds, ** = Recovery following a 2 h incubation in nutrient broth. n = 3.

According to the plate count results, the hypochlorite appeared to be effective in killing Pseudomonas attached to both rubber and teflon (Table 3). The other two enumeration methods do not implicate the same effect. The plate count method showed a log kill on rubber of 3.16, while the impedance and DEFT methods indicated a 1.40 and 0.45 reduction, respectively. The discrepancy in results was undoubtably due to injured bacteria which may not be accounted for. A two hour incubation in nutrient broth failed to recover these injured cells. The hypochlorite sanitizer reached the three log goal with Yersinia attached to teflon.

The acid anionic sanitizer had difficulty destroying bacteria attached to buna-N rubber (Table 4). The plate count reduction for Pseudomonas, Yersinia, and Listeria was 0.98, 1.34, 1.68, respectively. The impedance method indicated a similarly poor performance with an reduction of $\leq$ 1 for all of the tests organisms. This means that if this sanitizer were used in industry where attached bacteria were a significant problem, reduction in cell number would be approximately 90%. The peroxyacetic acid sanitizer shows similar trends usually with a higher reduction in bacteria attached to the rubber gaskets (Table 5).

Like the iodophor, the fatty acid sanitizer was more

effective in destroying viable cells on the teflon gasket surfaces (Table 6). The Quat sanitizer performed poorly against all organisms being tested (Table 7). This is surprising since QUAT's are normally more effective against Gram + organisms like Listeria. This effect could have been diminished because the glycocalyx layer inhibited direct contact between the sanitizer and the cellular membranes.

Of the three enumeration methods, the impedance detection method appeared to be the most useful way of quantifying cell numbers. It allowed the enumeration of bacteria that are reversibly and irreversibly attached. The DEFT method can be used to count the irreversible attachment in a separate step, but requires the removal of the reversibly attached cells. These removed cells have been subject to such harsh treatment and may cause inconsistencies in counts. The initial counts between the plate count method and the DEFT method seem to agree but there is wide disagreement between the two counts after treatment. Nonviable cells or injured, nonviable cells with some DNA remaining take up the acridine orange and fluoresce orange. There is no doubt that injured cells were present but attempts to recover them using a 2h. incubation in nonselective nutrient broth were unsuccessful. These injured bacteria may not be a serious problem in an actual

pipeline system that is allowed to completely dry during the intercycle period. If they are injured to the point where they cannot be recovered in nutrient broth, then chances are they will not recover in a more hostile environment. They may be a hazard, since they can provide a potential source of subsequent attachment or serve as a nutrient source to other cells in the biomatrix.

Once it was established that an increased resistance to sanitizers clearly existed, it was necessary to determine the extent of that heightened resistance. This was accomplished by doing a series of minimum inhibitory concentration (MIC) tests (Tables 8 & 9). Yersina and Listeria required a higher concentration of sanitizer before the minimum inhibitory level was reached. The same effect was not shown in the Pseudomonas results. This could be due to the harsh treatment required to remove the cells from the surface. Any disruption to the cell wall integrity would cause the sanitizers to be lethal at a much lower level. The higher minimum inhibitory concentrations for attached bacteria may help explain the inadequate log kill of some sanitizers. For example, the MIC for the acid anionic sanitizer increases from 200 to 500 ppm when the cells were attached. This clearly demonstrates the added protection the cells gain from the glycocalyx.

Table 8 - Minimum Inhibitory Concentrations for Pseudomonas fluorescens, Yersinia enterocolitica, and Listeria monocytogenes cell suspensions. (ppm concentration)

Sanitizer	Pseudomonas fluorescens	Yersinia enterocolitica	Listeria monocytogenes
IO	30	100	125
Cl	400	400	500
AA	150	550	200
PA	10	50	25
FA	90	400	50
Q	200	100	10

Table 9 - Minimum Inhibitory Concentrations for *Pseudomonas fluorescens*, *Yersinia enterocolitica*, and *Listeria monocytogenes* attached cells. (ppm concentration)

Sanitizer	Pseudomonas fluorescens	Yersinia enterocolitica	Listeria monocytogenes
IO	10	175	>175
Cl	300	500	>700
AA	150	>600	500
PA	10	75	50
FA	50	400	150
Q	100	10	25

CONCLUSIONS

Attached bacteria can be a serious problem in the food processing industry. Within the biomatrix, the bacteria are continually growing, multiplying, and being released into the environment. This could be an important source of product contamination. Since both pathogenic and spoilage bacteria have been shown to attach, their presence should not be overlooked. Prevention is the key to preventing biofilm build-up. Once a biofilm is firmly established, cleaning and sanitizing becomes much more difficult. Bacteria are less likely to survive the sanitation procedures if the proper concentration of sanitizer is used with the proper temperature and pH. With few exceptions, the sanitizers that were tested fell short of the three log reduction that was set. This does not prove these sanitizers to be ineffective. The suspension tests show that the sanitizers are working at the in-use concentrations. The results of this research have shown that the glycocalyx-covered cells are less susceptible to the same in-use concentrations. The entire cleaning/sanitizing regime must be examined in order to help prevent a biofilm build-up.

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VITA

The author, Tracy M. Mosteller, was born April 18, 1965 in Hopewell, Virginia. She is the daughter of Charles and Carol Mosteller. She grew up in Prince George, Virginia and graduated from Prince George High School in June of 1983.

In August 1983, she began attending Longwood College in Farmville, Virginia, working towards a degree in Biology. In May of 1987, she graduated from Longwood College with a major in Biology and a minor in Psychology.

She entered the Graduate School of Virginia Polytechnic Institute and State University in September, 1987 and is currently pursuing the Master of Science degree in Food Science and Technology.

Tracy is a member of Institute of Food Technologists (IFT), Phi Sigma, and the American Dairy Science Association.