SANITIZER EFFICACY TOWARDS ATTACHED BACTERIA IN A SIMULATED MILK PIPELINE SYSTEM USING PURE AND MIXED CULTURES

by

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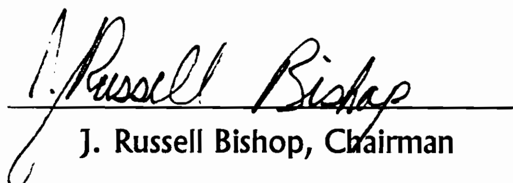

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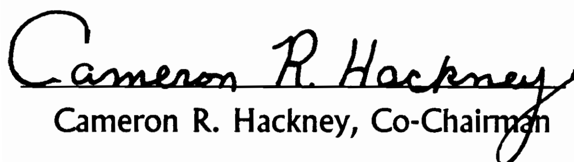

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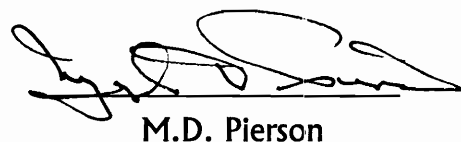

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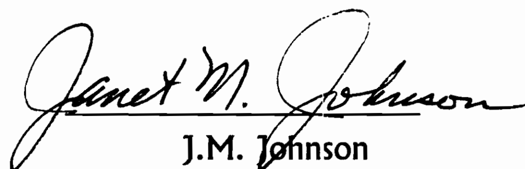

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(ABSTRACT)

The efficacy of six sanitizers, [chlorine (200 ppm), iodophor (25 ppm), acid anionic (200 ppm), peracetic acid (200 ppm), and fatty acid sanitizer (200 ppm)], was evaluated against bacteria attached to gasket materials. Pseudomonas fluorescens, Yersinia enterocolitica, Bacillus cereus, and Listeria monocytogenes were capable of significant attachment to both buna-N rubber and Teflon® gasket surfaces in either pure or mixed cultures. Differences in initial attachment rates were evident in a mixed culture of P. fluorescens, Y. enterocolitica, and Listeria monocytogenes in vitro. Sanitizer effectiveness depended upon the bacterium being enumerated, the type of surface, if the bacterium was attached in pure culture or as part of a mixed culture, and the system of evaluation, (i.e. whether or not sanitizer was used alone or as part of a cleaning system). Peracetic acid was the most effective. Removal of bacteria was more pronounced on the Teflon® surface with all sanitizers used. The cleaning system, which consisted of a pre-rinse

with warm water, application of the cleaning solution, post-rinse with warm water, and application of the sanitizing solution, allowed microorganisms to remain, when the bacteria were present as a pure culture; but, resulted in the complete removal of bacteria in mixed culture.

In a simulated milk pipeline system, significant differences ($P < 0.05$) in initial attachment of P. fluorescens, Y. enterocolitica, and B. cereus were observed with pure and mixed culture. The bacteria showed no preference for rubber or Teflon® gasket pieces in the simulated milk pipeline system. The sanitizers demonstrated no significant differences ($P < 0.05$) in effectiveness, providing a 1-2 log reduction in attached bacteria. Results from pure and mixed culture studies indicated that the cleaning system was effective in the removal of P. fluorescens from gaskets materials in a simulated milk pipeline system. Similar results were noted for Bacillus cereus in both pure and mixed cultures. Y. enterocolitica was found to be very resistant in pure culture. For example, 3.74 $\log_{10}/cm^2$ and 3.95 $\log_{10}/cm^2$ Yersinia remained attached to rubber and Teflon®, respectively, when exposed to 200 ppm chlorine in a simulated milk pipeline system. However, significant reduction ($P < 0.05$) of Y. enterocolitica was observed in mixed culture.

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INTRODUCTION

An effective cleaning and sanitizing regime is an important weapon for reducing bacteria to acceptable levels in dairy processing plants. Cleaning systems are implemented in processing plants to produce a wholesome, high quality product with an acceptable shelf-life. Clean-in-place (CIP) systems are commonly used to clean most of the food contact surfaces in dairy plants. The CIP system typically consists of the following steps: pre-rinse, 43°C for 1 min; cleaner, 63°C for 10 min; post-rinse 43°C for 1 min; and sanitizer 63°C for 2 min. Many pathogenic and spoilage bacteria are capable of attachment to materials commonly used in CIP 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 crevices which provide an excellent harborage for bacteria. In addition, these pits give the adherent cells protection from antimicrobial agents and surfactants. Pseudomonas fluorescens, Yersinia enterocolitica, Bacillus cereus and Listeria monocytogenes readily attach to both buna-N rubber and Teflon® surfaces. Once attached, a exopolysaccharide layer or glycocalyx forms effectively protecting them from antimicrobial agents that pass over the surface. Since pure

cultures of bacteria in suspension have no predilection for glycocalyx formation, sanitizer efficacy testing done in the laboratory with these glycocalyx-free suspensions could lead to false assumptions as to the sanitizer's true effectiveness under actual use conditions. In nature, few cells exist in a free floating state. In pure laboratory cultures, 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. Previous studies have indicated that sessile colonies are many times more resistant to antimicrobial agents than planktonic cells.

In addition, sanitizer efficacy tests do not provide an accurate representation of the entire cleaning system used in dairy processing plants. Each step in this system provides bacterial reduction and it is not left to the sanitizer to do the job alone. Since the glycocalyx covered colonies are more resistant to sanitizers, a study of sanitizer efficacy toward attached bacteria would help determine the sanitizers true capabilities. The use of a mixed culture of adherent cells provides greater insight into the interactions of bacteria and inert surfaces. Therefore, the objectives of this study were: (1) evaluate sanitizer efficacy of in-use concentrations toward a mixed culture of bacteria attached to gasket materials, (2) examine attachment on rubber versus Teflon® gaskets, (3) examine attachment of pure and mixed cultures in a simulated dairy pipeline system, (4) compare kill of attached bacteria to suspension tests, (5) determine the effectiveness of a cleaning system toward attached bacteria from pure and mixed cultures.

PSEUDOMONAS FLUORESCENS

I. History

Several decades ago milk was brought to the processing plant, pasteurized, bottled and delivered to the consumer's doorstep within 24 h of pasteurization, and usually consumed within 48 h. Today farm bulk tanks, every-other-day pickup at farms, five-day-a-week plant operation, grocery store purchase, and storage by the consumer have increased the age of milk before consumption (174). Milk may be 7-14 days old before consumption and this presents several new quality control problems.

Storage and handling of milk at refrigeration temperatures allows for the growth of psychrotrophic spoilage organisms. These organisms can grow at or below 7°C and produce extracellular enzymes that can damage the product. Gram negative bacteria are now the most commonly encountered spoilage organisms in the dairy industry. From an initial level of 10^4/ml, the psychrotrophic count can exceed 10^6/ml after 2 d of storage at 7°C (165).

II. Characteristics of the Organism

The family Pseudomonadaceae is represented by four genera Pseudomonas, Xanthomonas, Frateuria, and Zoogloea. Pseudomonas species are Gram negative,

aerobic, nonsporeforming rods that possess monotrichous or multitrichous, polar flagella. These catalase-positive rods are 0.5-1.0 μm in diameter and 1.5-5.0 μm in length. Growth of the organism occurs from 4 to 43°C with an optimum range of 25 to 30°C. These organisms commonly cause spoilage in a number of food products including fish, red meats, poultry, eggs, and dairy products. The addition of 0.05% potassium sorbate inhibits growth of P. fluorescens at pH 5.5 and 0.20% sorbate delayed growth at pH 6.0 (80).

Although they are inactivated after 30 min at 65.5°C, Pseudomonas is commonly found in pasteurized milk, probably due to post-pasteurization contamination (217). Growth of psychrotrophic organisms itself does not cause serious spoilage problems, however, they are able to produce extracellular enzymes that cause defects in milk. Two groups of enzymes of major economic importance are proteinases and lipases, including phospholipases which can act on micellar casein or on milkfat globules.

III. Proteinases and Lipases

The occurrence and types of heat-resistant bacterial enzymes depends on the type and number of microorganisms present, the production conditions, and length of storage time before processing (165). Inadequately cleaned and sanitized dairy farm and milk processing plant equipment are probably the major sources of contamination. Gram negative rods dominate the psychrotrophic

microflora of milk with Pseudomonas spp. comprising 50% of the genera, and with Pseudomonas fluorescens as the most common (165, 217). Sixty to ninety percent of the psychrotrophic bacteria are proteolytic or lipolytic and approximately 60-70% exhibit both types of enzymatic activity (165). Storage temperature, pH, and composition of the product influence enzymatic production. Production of extracellular enzymes occurs over a wide range of growth temperatures, but most researchers agree that optimum synthesis of P. fluorescens proteinases and lipases occurs between 20 and 25°C (153). Synthesis is inhibited at elevated growth temperatures. McKellar and Cholette (154) reported that P. fluorescens grown at 32°C are unable to produce proteinase. When resting cell suspensions were exposed to 32°C, synthesis of the enzyme halted, but resumed at 20°C after a 30 min lag period. Calcium is required for synthesis of extracellular proteinase of P. fluorescens. McKellar (153) observed that the addition of orthophosphate reduces proteinase synthesis and polyphosphates and EDTA prevents enzyme production without affecting growth.

Adams, et al. (1) reported the presence of proteinase-producing bacteria in 70-90% of raw milk samples obtained from one dairy farm and two dairy plants. Proteinase production per cell of P. fluorescens remains constant at 5 and 21°C, and 30% of maximum activity is retained at 7°C (153). Proteinases are very stable. After 3 months of storage at room temperature, significant amounts of enzymatic activity were retained.

The molecular weight of proteinases from P. fluorescens range from 40,000 to 50,000 and are most active at pH 6.5-8.0 (80). P. fluorescens AFT 36 proteinase demonstrates an optimum activity at pH 6.5, with 50% maximum activity at pH 5.5 and 8.0 (165). Stability of the enzyme decreases rapidly at pH values below 4.5 and above 10.5. This illustrates the serious implications of enzymatic activity in stored dairy products. These enzymes remain active over a wide pH range and retain activity at refrigeration temperatures.

Similar to proteinases from Pseudomonas, the lipase is relatively stable. Mottar (165) reported that for P. fluorescens spp. 27-41% of maximum activity remained at 20-25°C and relatively high activities of enzyme remain at 4°C for P. fluorescens spp. Lipases hydrolyze the triglycerides of milkfat into glycerol and free fatty acids. The C4 to C8 fatty acids are responsible for the development of rancid flavor, and the C10 and C12 fatty acids cause unclean, bitter, or soapy off-flavors (41).

IV. Effect on Milk Quality

The presence of heat-stable bacterial enzymes in milk which may survive pasteurization poses serious concerns due to the possibility of organoleptic defects and technological problems. The degradation of milk proteins can result in coagulation of milk, flavor defects, decreased yield, and poor shelf-life of dairy products. Researchers have observed that flavor changes can be detected when the

population of microorganisms is $> 10^8$/ml (180). In general, detectable changes are noticed at bacterial levels of 5 x 10^6 to 2.0 x 10^7 (165). Richter (185) reported that sensory changes are present when the population exceeds 10^6/ml. Still some researchers have found milk with higher levels that remains acceptable. The type of organisms and extracellular enzymes produced have a greater impact on quality than the actual bacterial number.

A. Proteinases

Micellar and soluble caseins are present in milk and are very susceptible to degradation by proteinases. Caseins are easily hydrolyzed by extracellular heat-stable proteinases, while whey proteins are only slowly degraded, if at all. Casein micelles are spherical (20-600 nm) particles. Adams, et al. (2) reported that k-casein was most susceptible to attack by Pseudomonas proteinases and could no longer be detected after at 5°C for 6 d. Coagulation of the milk could result from extensive k-casein hydrolysis, since k-casein serves to stabilize the micelles. Approximately 20% of milk proteins are whey proteins, mostly ß-lactoglobulin and α-lactalbumin. Most researchers agree that these whey proteins are not susceptible to degradation by proteinases produced by psychrotrophic bacteria (42).

Shelf-life of ultra high temperature (UHT) milk is greatly affected by the presence of heat-stable proteinases. A bitter flavor may develop in the milk and

gelation of the product may occur. Law, et al. (126) found that milk with 8 x 10^6 P. fluorescens/ml gelled after 8-10 weeks after UHT sterilization (140°C for 3.5 s) and storage at 20°C. Under the same conditions, gelation in milk occurred after 10-14 d in which 5 x 10^7 cfu/ml was present prior to sterilization. UHT milk appears to be much more sensitive to proteinases from P. fluorescens than raw milk. This could be due to differences in micelle integrity or to the presence of heat-labile proteinase inhibitors (165).

Loss of yield in cheese production has been reported when extensive bacterial proteinase activity has occurred. The flavor of ice cream and butter is not altered by the presence of proteinases. In ice cream, this could be due to the masking effects of sugar and vanilla, and in butter low protein levels (approximately 1%) are present.

B. Lipases

Lipid are classified into three groups: fats and oils; waxes; and phospholipids. In milk, fats and phospholipids are important considerations. Fats contain triglycerol esters of fatty acids that are easily hydrolyzed to glycerol and fatty acids. Phospholipids are complex compounds that are comprised of glycerol or other alcohols that are esterified by fatty acids and phosphoric acid (42). The lipids in milk are mostly triglycerides (98%). Ninety-five percent of milkfat exists in the globular form with an outer membrane (milkfat globule membrane) which is

comprised of proteins, phospholipids, glycolipids, sterols, and glycerides.

Spoilage of pasteurized milk due to lipolytic activity is not commonly reported. Defects caused by this type of activity are mainly reported in products that are stored for extended periods of time. Several psychrotrophic bacteria produce extracellular phospholipase. These enzymes are especially important in raw milk because they degrade the phospholipids of the milkfat globule membrane, increasing the susceptibility of the milkfat to lipolysis (165).

BACILLUS CEREUS

I. History

Bacillus cereus has been linked to foodborne illness since 1950 when Hauge published the results of his investigation of four outbreaks in Norway (89). The incidents were similar and approximately 600 people were affected. In one outbreak at a hospital, the menu included a meat dish with vegetables, and chocolate pudding with vanilla sauce for dessert. Both the pudding and vanilla sauce had been prepared the previous day and stored at room temperature in a large container. Of the 80 people who ate the meal, 61 experienced abdominal pain, profuse watery diarrhea, rectal tenesmus, and nausea without vomiting. The average time before onset of symptoms was 10 h. The vanilla sauce was analyzed and found to contain 2.5×10^7 to 1.1×10^8 B. cereus/ml. Even though such high numbers of the organism were found the product displayed little change in color, odor, taste, or consistency. Spores of B. cereus, up to 10^4/g, were found in the cornstarch used to make the vanilla sauce.

Since 1950, there have been an increasing number of reports that implicate this organism as the causative agent. The majority of these reports came from Scandinavian and Eastern European countries. In Hungary, B. cereus was reported as the third leading cause of bacterial food poisoning between 1960 and 1968.

Researchers indicate that this is probably due to cultural food preparation and handling techniques (89). Hungarian meat dishes are frequently well-seasoned with spices that often contain relatively high numbers of spores. Cooking was usually insufficient to destroy the spores, and improper storage allowed the surviving spores to germinate and proliferate. The first well documented case in the United States was reported in 1970.

II. Characteristics of the Organism

Bacillus cereus is a large Gram positive, facultative aerobe, sporeforming rod. Vegetative cells are 1.0-1.2 μm in diameter and 3.0-5.0 μm in length. The spores are ellipsoidal, central, or paracentral. Growth range for the organism is 10 to 48°C with and optimum of 28 to 35°C. Raevuori and Genigeorgis (182) reported that the pH range for B. cereus growth was 4.9-9.3 and the minimum a_w was 0.95. The authors also observed that growth of the organism occurs at 7.5% salt, but not 10%.

Murrell (168) indicated that Bacillus can utilize glucose, fructose, and trehalose, but not pentoses and many of their sugar alcohols. Some strains attack sucrose, salicin, maltose, mannose, glycerol, m-inositol, and lactose. A few strains are urease positive, and most strains actively hydrolyze starch, casein, and gelatin.

Nisin, 0.2% sorbic acid, and 0.4% potassium sorbate have an inhibitory

effect on B. cereus (114). Johnson (114) also reported that the organism is susceptible to aureomycin, dihydrostreptomycin, terramycin, bacitracin, oxytetracycline, chloramphenicol, and gentamicin. Slight inhibition was observed with neomycin, cloxacillin, ampicillin, and penicillin.

The organism is commonly isolated from soil and vegetation. Two types, diarrheal and emetic, of foodborne illness are possible as a result of two distinct enterotoxins. This is evident in the different incubation periods for each illness. Diarrheal illness usually strikes within 10-13 h of consumption, while the emetic type causes symptoms in 1-5 h.

III. Growth and Survival

The presence of B. cereus in milk was well established before the pathogenicity of the organism was known. It causes a defect known as "bitty" cream or sweet curdling of milk. Billing and Cuthbert (14) surveyed dairy farms and found that B. cereus was commonly present, especially on the milk cans where 10.5% showed more than 5×10^3/can. Milk can contamination is not significant today due to the use of large bulk storage tanks, but does demonstrate the presence of B. cereus in the environment and gives rise to concerns that dairy products or products made from milk could support the growth of B. cereus (49). Davies and Wilkinson (49) suggested that milk contamination could be especially important in the shelf stable pudding products.

Ahmed, et al. (3) collected 400 samples of milk and milk products over a 5 month period from retail outlets in Wisconsin. B. cereus was isolated from 9, 35, 14, and 48% of raw milk, pasteurized milk, Cheddar cheese, and ice cream samples, respectively. The authors noted that the number of B. cereus present did not exceed 100/ml in raw milk, 1000/ml in pasteurized milk, 200/g in Cheddar cheese, and 3800/g in ice cream.

Mikolajcik, et al. (158) studied the fate of B. cereus in cultured skim milk and Cheddar cheese. They found that as the acidity of skim milk increased, the number of vegetative cells decreased, while the spore count remained constant. At pH 5.0, spore germination ceased. In Cheddar cheese manufacture, the authors observed that B. cereus grew rapidly after cooking to the end of the milling process. They indicated that B. cereus survived in the spore state in Cheddar cheese throughout the 52 week storage period.

Kim and Goepfert (121) studied the occurrence of B. cereus in selected dry food products. The results of their study indicated that 25% of 170 samples of dried food products are positive for B. cereus. The levels of organism present did not exceed 4×10^3. Blakely and Priest (17) suggested that soaking dried legumes for extended periods of time before cooking could be hazardous if B. cereus is present. Their study showed that 56% of surveyed pulses and cereals were contaminated with B. cereus. Several types of legumes are typically soaked 18 to 24 h before cooking, which could lead to prolific growth of B. cereus.

Nester and Woodburn (169) isolated B. cereus from Mexican-style beans in restaurant settings. The authors indicated that these beans were typically prepared in bulk, improperly cooled, and improperly reheated before placement on the steam table. B. cereus reached levels high enough to cause illness in 4 h and obvious signs of spoilage did not occur until 12h.

IV. Gastroenteritis

A. Diarrheal Type

This type of illness has an incubation period of 10-13 h, and is characterized by symptoms of acute colitis or enterocolitis. Symptoms include abdominal pain, profuse watery diarrhea, and rectal tenesmus. Nausea is moderate and vomiting rarely occurs. Illness is very similar to Clostridium perfringens in terms of symptoms and incubation time (114).

B. Emetic Type

From 1971-1979, 110 cases of emetic food poisoning from B. cereus were reported in the United Kingdom. Ninety of the 110 cases occurred during the months of June to September. The symptoms of illness included acute attacks of nausea and vomiting 1-5 h after ingestion. 108 of the 110 cases were linked to the consumption of cooked rice. 104 of the 108 rice-associated incidents involved boiled or fried rice from Chinese restaurants (89).

These restaurants boil the rice in bulk and allow it to air dry and cool down at room temperature, and then it is reheated or fried quickly with a beaten egg. Usually the rice is left at room temperature overnight, but it can be stored from a few hours to 3 days. Spores of B. cereus can survive these boiling and frying procedures. Vegetative cell growth is rapid at room temperature and is enhanced by the addition of beef, pork, or egg.

V. Epidemiology

Foods most often implicated in outbreaks include meat and meat products, puddings, vanilla sauce, cream pastry, vegetables, mashed potatoes, soups, and most commonly, rice (61). The illness is often confused with S. aureus intoxication due to the rapid onset of symptoms. The first reported outbreak in the United States involved meatloaf. Members of a California fraternity experienced abdominal pain, diarrhea, nausea, and some vomiting 10 h after consumption. Analysis of the meatloaf found 7.0×10^7 B. cereus/g (157). Typically B. cereus is found in low numbers in foods and growth must occur in order to reach levels necessary to cause foodborne illness. The infective dose is usually at least 10^6 cell/g, but more commonly 10^7 to 10^8 are typically found (168). The sudden onset of symptoms, short duration, and lack of fever suggests an intoxication. Still, very little is known about the mechanisms of pathogenicity. Symptoms of the two types of illness are caused by separate and distinct toxins

(114). B. cereus also produces phospholipase C, hemolysins, and mouse lethal toxins, which are distinct from the illness causing toxins. The hemolysin and lethal toxin have been ruled out as causes of illness because they do not cause fluid accumulation in ligated ileal loops of rabbit.

The diarrheal toxin has been characterized as a protein with a molecular weight of 50,000 and an isoelectric point of 4.9. Recent research indicates that this toxin is composed of two components. Toxin production occurs in a pH range of 6.0 to 8.5, with an optimum of 7.0 to 7.5 (114). It is produced during log phase growth at 18 to 35°C. The toxin is unstable during storage at 4 or 25°C and is inactivated at 56°C for 5 min (91).

The emetic toxin is different from the diarrheal toxin, with a molecular weight of 1,000 to 5,000 daltons. It is stable at 126°C for 90 min, and storage at 4°C for 2 months. The toxin is stable in the pH range of 2-11 and trypsin and pepsin do not inhibit the emetic response.

VI. Identification

Typical enumeration and isolation procedures involve an agar plate method. The characteristic reactions of various plating media include hemolytic, lecithinase activity, or fermentation properties. An inhibitor is usually included to inhibit competitive microflora. The procedures are simple and rapid, but presumptive.

Mossel, et al. (163) developed mannitol egg yolk polymyxin (MYP) agar to

isolate B. cereus from food. Polymyxin prevents growth of competing microflora and egg yolk and mannitol provide differential reactions. Typical colonies appear rough with a violet-red background, surrounded by white precipitated egg yolk. Typical colonies may be confirmed by biochemical or fluorescent antibody tests. Murrell (168) indicates that the minimum biochemical tests to identify B. cereus include: catalase, (+); Voges-Proskauer, (+); anaerobic growth, (+); growth at 50°C, (-); acid and gas from glucose (-); growth at pH 5.7, (+).

VII. Prevention

The widespread distribution, ability of spores to survive long-term storage in dried products, and thermal resistance are factors that emphasize the need to take control measures when dealing with food products. Control measures should be taken to prevent growth during processing and subsequent storage. As with any food product preventative measures include: preparing small quantities; keep prepared food hot (>55°C); cool unused portions quickly; and reheat thoroughly before serving. Reheating will not destroy toxins that were previously produced, therefore, preventing growth of the organism is the most useful weapon in controlling illness due to B. cereus (114).

LISTERIA MONOCYTOGENES

I. History

Listeria monocytogenes is a facultative intracellular bacterial pathogen that has become increasingly recognized as an important source of human infection. Descriptions of the Gram positive rods which were suspected to cause listeriosis were reported as early as 1891 (135). Lovett (135) reported that it was Murray, et al. who were credited with the discovery in 1926 of an organism which was a pathogenic for laboratory animals. Since monocytosis was a key symptom, the organism was named Bacterium monocytogenes. B. monocytogenes was isolated from individuals exhibiting symptoms of mononucleosis and from infants with systemic infection. The name Listeria monocytogenes was proposed in 1940 and later accepted. Research conducted before 1960 was based on animals models and was principally directed by M.L. Gray, et al (135). They studied listeriosis in sheep, cold enrichment, oblique lighting for detecting L. monocytogenes on isolation agar, and variation in colony morphology (135).

It has been only within the last two decades that L. monocytogenes has emerged to become a serious concern to food scientists. Cox (43) discussed why "new" pathogens emerge and indicates that it is not a result of any one factor but a complex mutual interaction of many components. The author discussed the

following 7 basic reasons for the emergence of "new" pathogens: 1) changes in eating habits; 2) changes in perception and awareness of hazards; 3) demographic changes; 4) changes in primary food production; 5) changes in food processing technology; 6) changes in handling and preparation practices; and 7) changes in the behavior of microorganisms.

II. Characteristics of the Organism

L. monocytogenes is a small, short, Gram positive coccobacilli, 0.4-0.5 μm in diameter and 0.5-2.0 μm in length. It has a tendency to form short chains of three to five organisms in young cultures, lying parallel or in a "V" shape. Older cultures, 3-5 days old, appear more filamentous and may be Gram variable (115, 135).

Listeria are flagellated and motile in a characteristic tumbling or slightly rotating fashion, however the degree to which flagellation occurs is temperature dependent. At 20-25°C, L. monocytogenes is actively motile by means of four peritrichous flagella. At higher temperatures motility is suppressed, although most cultures grown between 30 and 37°C still demonstrate some motility by means of one polar flagellum. Listeria species grow well on simple nutritional media with a pH of 5.0-9.0. On the clear colorless nutrient agar, Listeria colonies appear translucent, and blue-green when viewed by 45° incident transmitted light.

All species produce acid but no gas from the fermentation of dextrose,

esculin, or maltose. Most clinical strains do not ferment mannitol. In addition, L. monocytogenes produces acid but no gas from rhamnose. Another important characteristic of this organism is the production of hemolysis on blood agar and a more definitive adaptation of the same reaction called the CAMP test (135).

Growth of L. monocytogenes occurs over a temperature range of 2.5-44°C, although growth at 0°C has been reported (135). The organism can withstand repeated freezing and thawing. This ability to grow well at refrigeration temperatures makes this pathogen especially interesting to food microbiologists. Listeria have simple nutritional requirements and can grow well on tryptose agar and sheep blood agar. This organism is facultatively aerobic to microaerophilic and catalase positive. Growth of most species is improved when incubated under reduced O_2 conditions and a CO_2 concentration of 5-10%.

Based on their O (somatic) and H (flagellar) antigens, 13 serotypes of L. monocytogenes have been identified. In the United States and Canada, serotype 4b is the predominant strain. Presently, three strains (1/2a, 1/2b, 4b) cause at least 90% of all clinical Listeria infections throughout the world (115).

III. Growth and Survival

Processing and handling techniques such as pasteurization, addition of antimicrobial agents, and/or refrigeration are employed to prevent growth and survival of microbial pathogens. L. monocytogenes differs dramatically from other

bacteria in its response to these control measures. Special consideration should be given to this organism in order to prevent its survival and subsequent growth in consumer food products.

L. monocytogenes is one of a few foodborne pathogens that exhibit grow at refrigeration temperatures (51). Growth occurs in a temperature range between 3°C or below and 45°C, with optimal growth occurring between 30 and 37°C. The minimum pH at which L. monocytogenes can grow is still unclear. Some reports indicate that the organism cannot grow below pH 5.6; however more recent studies reveal that listeriae can grow at pH 5.0 (51). The organism grows well at alkaline pH and growth can be seen in liquid media up to pH 9.6.

Reports indicate that L. monocytogenes is quite salt tolerant. Doyle (51) reported that Listeria are capable of growth in 10% NaCl and can survive up to 1 year in 16% NaCl at pH 6.0. The author also stated that researchers report that the organism can survive for 15 days at 37°C in 10.5% NaCl and 5 days in 20-30% NaCl. L. monocytogenes survived for more than 100 days in 10.5-30% salt at 4°C. Studies with sodium nitrite show that L. monocytogenes is not inhibited by currently allowable levels unless there is an interaction with other antimicrobial agents such as salt (51).

Nisin at 16 IU/ml is inhibitory to L. monocytogenes; however, after a 30 h lag period, the organism grew well (135). Recent studies demonstrated that L. monocytogenes strain Scott A serotype 4b can grow in the presence of 2,000 IU

of nisin/ml of tryptose broth at 37°C, after a lag phase of a few days. Other strains of L. monocytogenes were less resistant than Scott A but grow was evident in 500 IU of nisin/ml, after an initial lag period.

Several studies suggested that L. monocytogenes may not be destroyed by the minimum heat treatment used in the pasteurization of milk if present in high numbers. Epidemiological investigations of a listeriosis outbreak in Massachusetts in 1983 indicated that a specific brand of pasteurized milk was responsible. The implicated milk came from dairy farms on which listeriosis in dairy cows was known to have occurred at the time of the outbreak. The plant where the milk was processed revealed no evidence of improper pasteurization. Researchers suggested that the ability of L. monocytogenes to exist as an intracellular parasite may have increased the organism's ability to survive pasteurization (51). Lovett, et al. (136) indicated that the incidence of L. monocytogenes contamination in raw milk may range from 0-7%. A study conducted in Spain found that more than 21% of pasteurized milk from one Madrid Dairy plant was contaminated with the Listeria (20). These studies the potential for L. monocytogenes contamination of both raw and pasteurized milk. Cheddar cheese made from raw milk must be aged for a minimum of 60 days before sale in order to eliminate pathogens. Ryser and Marth (190) manufactured cheese using pasteurized whole milk inoculated with 5 x 10^2 L. monocytogenes/ml. Bacterial counts remained relatively constant during the manufacture process. After 14 days of ripening, L. monocytogenes reached a

maximum of 5 x 10^3 cells/g and then began to decrease during further ripening at 6 or 13°C, with L. monocytogenes detected in cheese at 154-434 days of ripening. These results indicated that the 60 day holding period for raw milk cheddar cheese is not adequate for the elimination L. monocytogenes from cheese.

Several recalls of Camembert and Brie cheese occurred during the late 1980's because of L. monocytogenes contamination. These soft, surface-ripened cheeses have a relatively high pH (>6.0). Ryser and Marth (191) manufactured Camembert cheese from milk inoculated with an initial level of 5 x 10^2 cfu/ml. Similar to the Cheddar cheese, counts initially increased to about 5 x 10^3 cells/g. The number of Listeria remained constant during the first 25 days of ripening at 6°C. After 56 days of storage, the number of cells had increased to 1 x 10^7 cells/g. This research demonstrated the ability of L. monocytogenes to proliferate in the soft, surface-ripened cheeses of high pH. Listeria may survive in the hard, aged cheeses with pH <5.5 but do not increase in number.

Ryser, et al. (192) manufactured cottage cheese by the short-set method using skim milk inoculated with 10^4-10^5 cells/ml. During the manufacture process, cell numbers remained relatively constant until after cooking of the curd, 57.2°C for 30 min. After cooking the curd (pH 4.7), L. monocytogenes could not be detected by a direct plating procedure (< 10 cell/g) and was inconsistently detected by an enrichment procedure, suggesting that small numbers survived the manufacturing process.

Doyle, et al. (54) used skim milk, concentrated (30% solids) and unconcentrated, inoculated with 10^5-10^6 Listeria/ml. The milk was spray dried to 3.6-6.4% moisture, and stored in moisture resistant packages at 25°C for up to 16 weeks. They observed a 1-1.5 x $\log_{10}$ decrease of cells/g during drying and found that the numbers of L. monocytogenes gradually decreased during storage, with a decrease of 10^4 cells/g occurring during the 16 weeks of storage.

Studies of ground beef stored at 4°C indicated that the number of L. monocytogenes remained constant at 10^5-10^6 cells/g throughout 14 days of storage (135). Similar results were found with lamb and beef stored for 14 days of storage; however significant growth in the sarcoplasmic proteins of pork held under the same conditions occurred within 6 days of storage (5). These differences in the growth pattern of L. monocytogenes on different types of red meat are still not fully understood.

Researchers isolated L. monocytogenes from 57% of fresh and frozen poultry samples. Reports indicate that about 15% of the oven ready poultry samples contain L. monocytogenes. A survey in the United States indicated that about 70% of ground beef, 43% of pork sausage, and 48% of poultry are potentially contaminated with L. monocytogenes (135). Fresh vegetables, such as cabbage, lettuce, celery, and tomatoes, have been implicated as sources of human infection. Shellfish may be especially susceptible to contamination since they are filter-feeders. L. monocytogenes is able to successfully compete with other

microflora, survive, and grow at refrigeration temperatures (20).

IV. LISTERIOSIS

Clinical manifestations of listeriosis range from a mild flu-like illness to fulminant neonatal listeriosis associated with mortality rates of 54 to 90%. In adults, the major infections are meningitis (55%), primary bacteremia (25%), endocarditis (7%), and nonmeningitic central nervous system infection (6%) (115). More than half of these patients have pre-existing conditions such as malignancy, alcoholism, cirrhosis, diabetes, or vasculitic or are receiving immunosuppressive drugs. Human listeriosis is commonly associated with formation of miliary granulomas (mass of inflamed tissue made up of many small lesions) and focal necroses or by suppuration (formation of pus) in the infected tissues (145). Size, number, and sites of lesions may vary from person to person, and are influenced by the infective dose, source of infection, and age and health status of the host.

The most distinctive form of infection is the genital tract illness in pregnant women with subsequent infection of the offspring. The infection may occur anytime during pregnancy, but are more common during the third trimester. Usually the mother remains symptomless or has only a self-limited influenza-like illness during the last trimester of pregnancy with symptoms including fever, chills, headache, backache, and discolored urine. Pharyngitis, diarrhea, and pyelitis

(inflammation of the pelvis of the kidney) have also been reported.

Two types of infection are possible for the infant. The early type, granulomatosis infantiseptica, results from transplacental transmission and is evident within 2 days of birth. This type of infection is likely to be bacteremia, which can lead to the presence of L. monocytogenes in fetal urine. Urine is discharged into the amniotic fluid, where it is aspirated by the fetus, leading to widespread involvement of the respiratory and gastrointestinal tracts (145). Symptoms of listeriosis of the infant commonly include respiratory distress, heart failure, difficult and forced respiration, cyanosis, refusal to drink, vomiting, convulsions, soft whimpering, early discharge of meconium, and mucus stools (145). Transplacental infection of the fetus typically leads to spontaneous abortion, premature birth, stillbirth, or death within a short period after birth may occur. If born alive, the infant experiences symptoms of septicemia usually within a few hours, often followed by fetal distress, pneumonia, diarrhea, seizures, and maculopapular skin lesions on the body and legs. This type of infection is associated with a very high mortality rate.

The second or late type of neonatal listeriosis usually appears after the fifth day following birth. The infant usually demonstrates symptoms of meningitis, which include shallow and rapid breathing, slight cyanosis, lethargy, fever and anorexia. Infection is acquired either during or after birth rather than in utero. The mother usually experiences no symptoms. About 10% of neonatal meningitis

is due to L. monocytogenes (115).

In the adult, meningitis is the most commonly recognized form of listeriosis and a leading cause of bacterial meningitis in cancer and renal transplant patients. The illness begins with mild flu-like symptoms and progresses into headache, pains in the legs, chills, pyrexia, neck stiffness, nausea, vomiting, and photophobia. Clinically, listerial meningitis cannot be distinguished from meningitis caused by other bacterial infections of the meninges (145). The mortality rate among all patients with meningitis is 30%, but among cancer patients the mortality rate is approximately doubled (115).

L. monocytogenes produces toxins, hemolytic and lipolytic, which may be involved in the disease process. Also, the cell wall of L. monocytogenes appears to contribute to its pathogenicity, although a variety of constituents seem to contribute to the total effect. These factors include: 1) a water soluble toxic polysaccharide able to produce lymphopenia and granulocytosis; 2) protein and carbohydrates which are antigenic, pyrogenic, and able to induce lymphopenia and granulocytosis; and 3) pyrogenic, fractionated glycine lysate which increases the virulence of L. monocytogenes, and caused granulocytosis (145).

Treatment of listeriosis generally includes administration of penicillin or ampicillin. Erythromyosin and tetracyclines are also effective treatments. Cephalosporins have variable activity and limited penetration of the meninges.

V. VIRULENCE FACTORS

Due to its widespread distribution, it is probable that we ingest virulent and avirulent Listeria species very frequently, if not daily. The virulence of L. monocytogenes is complex and apparently due both to soluble and cell surface antiphagocytic components. The soluble components excreted during growth include hemolytic and lipolytic antigens that appear to be an important factor in the pathogenesis of the infection. The function of the Listeria hemolysin may be to disrupt membranes, especially those of the phagocytic vacuole and the lysosomes. Both hemolytic and nonhemolytic strains are taken up by macrophages, but only the hemolytic cells survive.

The exact function of the lipolytic antigen remains uncertain. A correlation exists between hemolysin production, lipolytic activity, and virulence. All avirulent and most nonhemolytic-producing strains show either diminished or no lipolytic activity (115). The lipolytic and hemolytic antigen appear to be two individual components rather than a single antigen with both hemolytic and lipolytic activity.

Schlech (199) suggests that the following factors play an important role in determining the virulence of L. monocytogenes:

- Serotype is perhaps the best characterized virulence factor of L. monocytogenes. Serotypes 1 and 4 are responsible for most invasive human infections.

- Lysogenic phages are present in all species of Listeria, and most isolates

can be phage typed.

- Large outbreaks of listeriosis have been attributed to nonplasmid-bearing strains, and multiple plasmid types may occur during the same epidemic. Therefore, it seems unlikely that plasmids code for differences in virulence, as in Yersinia species.

- Biotyping of Listeria species has been more useful than serotyping and phage typing in identifying virulent and avirulent strains of Listeria. The most useful tests include xylose acidification, which does not occur in virulent organisms, and the CAMP test, which can identify virulent L. monocytogenes.

- Phenotypic expression of "rough" or "smooth" colony morphology has been shown to influence virulence in such enteric pathogens as salmonellae.

- Hemolysins of L. monocytogenes are important markers for distinguishing virulent strains. However, they have a variable expression within individual pathogenic strains. Hemolytic organisms tend to exhibit smooth colony formation. The loss of hemolysis through mutation can result in a loss of virulence. Despite the clear association with virulence, the significance of hemolysin production is still unclear.

- Virulent strains of L. monocytogenes produce increased levels of superoxide radicals and hydrogen peroxide as compared to avirulent strains. The production of oxygen radicals may serve to improve the intracellular survival of L. monocytogenes in macrophages.

VI. EPIDEMIOLOGY

L. monocytogenes is paradox of nature; sporadic in occurrence and worldwide in distribution. It has been isolated from soil, dust, animal feed, water, sewage, and almost every type of animal tested, including asymptomatic human carriers. Tested species include 42 domestic and wild mammalian species, 22 avian species, fish, ticks, a fly, and crustaceans. Also, it is commonly found in plant and soil samples and in animal feces. Currently, scientists believe that L. monocytogenes is a saprophytic organism, which exists in a plant-soil environment and can be contracted by humans and animals from a variety of sources. For many years L. monocytogenes was ignored by food microbiologists. This has changed recently due to Listeria's emergence as a foodborne bacterial pathogen. Recent epidemiological evidence has implicated food as the most common vehicle for transmission of human listeriosis in both adults and neonates. The only known example of person-to-person transmission is transplacental infection of the fetus or infection during delivery through contact with infective secretions (135).

In the United States, the number of human listeriosis cases appears to be increasing. During 33 years (1933-1966) there were 731 confirmed cases in the United States; subsequently, during the next 3 years (1967-1969), 255 cases were reported to the Centers for Disease Control (CDC), and these were from only 35 states (5). Some of this apparent increase may be a result of greater awareness and improved identification methods. In addition, some of this increase

is related to the rising numbers of immunocompromised patients who are exposed to infectious complications. The age distribution of the United States may also become a factor since most listeriosis cases occur among neonates and the elderly.

Cox (43) discusses some observations on the epidemiology of listeriosis:

- Disease is rare but the organism is common in the environment.
- Outbreaks are rare; the disease usually occurs as sporadic cases.
- The disease is linked to the consumption of food.
- The disease occurs in predisposed individuals and has a 30% mortality rate.
- L. monocytogenes can be carried without effect, even in sensitive people.
- L. monocytogenes can be carried without effect for varying periods prior to onset of symptoms.
- The incidence of listeriosis peaks in summer and winter.
- Incidence is increasing in some countries.
- Diarrhea, nausea, and vomiting sometimes occur prior to onset of illness.
- Outbreaks can involve different serotypes.

The author also stated that the severity of the illness may depend on the degree of infectivity, the health of the host, and the virulence of the organism. L. monocytogenes can be present in the feces of both healthy and sensitive humans, sometimes at high levels without causing disease.

There have been three well-investigated foodborne listeriosis outbreaks in the last decade. In Nova Scotia, 34 cases of perinatal listeriosis and 7 cases of adult disease occurred between March 1 and Sept. 1, 1981. The outbreak strain was identified as L. monocytogenes serotype 4b, and the investigation implicated coleslaw as the vehicle. Cabbage used to make the coleslaw was fertilized with sheep manure. Investigations at the farm showed several of the sheep had been

infected with Listeria (145).

Between June 30 and August 30, 1983, 49 individuals in Massachusetts became infected, with a mortality rate of 29%. Thirty-two out of forty L. monocytogenes isolates tested were serotype 4b with the remaining isolates were 1a, 3b, and 4ab. Epidemiological investigations traced the source of infection to a particular brand of milk. Dairy plant inspections revealed no signs of equipment failure or improper pasteurization. L. monocytogenes was not isolated from any pasteurized milk samples but was identified in 12% of raw milk samples and in 2 of 14 milk filters from the farms supplying the milk (135).

Between January 1 and June 14, 1985, 86 cases of listeriosis were reported in Los Angeles and Orange Counties, California. Most of the patients were mother-infant pairs. Forty-two of the neonatal patients developed symptoms of listeriosis within 24 h after birth. The majority of patients cultured positive for L. monocytogenes serotype 4b. Investigations implicated a Mexican-style soft cheese manufactured by a plant in southern California. Researchers found evidence of improper pasteurization of the milk. L. monocytogenes of the outbreak type was isolated from the cheese and the manufacturing plant environment (145).

VII. IDENTIFICATION

The FDA method for recovering Listeria from milk and dairy products and

non-dairy products involves enrichment with modified Listeria enrichment broth (milk and dairy products) or Listeria enrichment broth (non-dairy products). After enrichment, oxford agar (OXA) or LPM agar is used. The USDA/FSIS recommends the use of UVM modified Listeria enrichment broth for poultry and meat products. Follwing this primary enrichment, a secondary enrichment step with Fraser broth is used. Next, modified oxford agar (MOX) is used for isolation.

After cold enrichment and plating on selective media, colonies are streaked onto Trypticase soy agar supplemented with 0.6% yeast extract (TSA-YE) to check for purity. Typical colonies from TSA-YE plates are examined for the following reactions: Gram stain, catalase production, motility in SIM agar at room temperature, hemolysis on sheep red blood plates, nitrate reduction, urease production, H_2S production in TSI, MR-VP reaction, and the ability to use the following carbohydrates with acid production but no gas: esculin, maltose, mannitol, rhamnose, and xylose (135). Isolates giving the proper response are serotyped and tested for mouse lethality. The CAMP test is also used to examine hemolysis when the species identification is unclear.

The CAMP test is a definitive analysis for identifying ß-hemolysis activity because metabolites of ß-hemolytic S. aureus and Rhodococcus equi are used to enhance the hemolytic reaction of Listeria ssp. in a characteristic pattern. In the test, S. aureus and R. equi cultures are streaked vertically on sheep blood agar and separated by 4.5-5.0 cm. The Listeria ssp. test cultures are streaked horizontally

between the vertical streaks without touching them. The hemolytic reactions of L. monocytogenes and L. seeligeri are enhanced in the vicinity of the S. aureus streak but not in the vicinity of the R. equi. L. ivanovii cultures are more hemolytic in the vicinity of the R. equi streak but are unaffected by the S. aureus streak (135).

YERSINIA ENTEROCOLITICA

I. History

Yersinia enterocolitica was first described in 1939 (124). The bacteria that were isolated from human infections that resembled Bacillus lignieri and Pasteurella pseudotuberculosis. During the next two decades, reports from the New York State Department of Health noted sporadic isolation of similar organisms, which were called Bacillus enterocoliticum. Like many other psychrotrophic pathogens, its significance was not realized until many decades later. In 1961, Dickenson and Mocquot described Gram negative bacteria isolated from swine that included some atypical Pasteurella pseudotuberculosis strains, which they called "type b" (198). The genus *Yersinia* was created to honor Yersin, a French bacteriologist. In 1894, Yersin isolated the plague bacillus during an epidemic in Hong Kong. Based on numerical taxonomy studies, it was recommended that Pasteurella pseudotuberculosis, Pasteurella pestis and Pasteurella "X" be included in the genus Yersinia, and that this genus should be placed in the family Enterobacteriaceae. The species Y. enterocolitica once contained a number of biovars but the most recent volume of Bergey's Manual of Determinative Bacteriology divides it into several species, including Y. enterocolitica, Y. intermedia, Y. fredriksenii, and Y. kristensenii (52). The incidence of yersiniosis in the United States has remained far lower than that in many other countries, including neighboring Canada. In

1966, there were only 23 cases of yersiniosis reported worldwide (124). But with greater awareness, Y. enterocolitica became increasingly implicated in foodborne illnesses. From 1967-1977, there were 200,000 reported cases in Denmark, 2,000 in Belgium, 1,000 in Canada, and 300 in the United States (52).

II. Characteristics of the Organism

Y. enterocolitica is a Gram negative pleomorphic (ovoid to rod shaped) facultative anaerobe. This bacterium is motile with a peritrichous flagella when the growth temperature is < 30°C (52). Organisms classified as Y. enterocolitica are serologically heterogeneous. Twenty-seven serotypes have been identified on the basis of their O and H antigens (116). Winbald described a system, which used heat stable "O" (somatic) antigens for serotyping Y. enterocolitica (198). The first typing system was based on eight antigenic factors. Wauters expanded this number 17, then to 34, and later to the present 57. It has been suggested that a revised scheme based on 20 factors be adopted for Y. enterocolitica (198). Certain serovars are consistently associated with human illness. Serovars O3 and O9 are prevalent in Europe, Canada, and Japan. In the United States, serovar O8 is commonly found.

III. Growth and Survival

Yersinia enterocolitica is a member of the Enterobacteriaceae and is often associated with the gastrointestinal tract of warm-blooded animals. Y. enterocolitica is a unique pathogen because it is psychrotrophic with a growth range of 0-44°C with an optimum of 32-34°C. This bacterium grows well on conventional enteric agar at 22-25°C, but will not grow at 35-37°C. Inhibited growth on selective media at 35-37°C is due to increased toxicity of selective agents, and an additional calcium requirement in plasmid-positive strains. Even a nonselective, nutritious medium such as Trypticase soy-0.6% yeast extract agar will not provide for good growth of plasmid-positive Y. enterocolitica at 35-37°C unless the medium has been supplemented with 2.5 mM calcium (198). Y. enterocolitica will grow in a simple glucose-salts medium at temperatures $< 28°C$, but very slowly. Y. enterocolitica can grow under alkaline conditions, but cannot tolerate acid pH well. The range for growth is pH 4.6-9.0 with an optimum of 7.0-8.0. Growth of the organism is inhibited at 7% salt.

Y. enterocolitica has a high tolerance for surface-active agents, especially bile salts. This tolerance appears to be greater in pathogenic strains. Irgasan (2,4,4'-trichloro-2-hydroxydiphenyl ether) is tolerated at concentrations that will inhibit other Enterobacteriaceae (195). Dye tolerance does not differ from that of most other Gram negative bacteria.

Hanna, et al. (101) demonstrated extensive destruction of Y. enterocolitica

on the surface of beef during frozen storage. With an initial inoculum of 10^4 cells/g, the authors found no survivors of strains 1157 and 1049 after 2 and 4 weeks of frozen storage (-23°C), respectively. In the same study, the authors showed that Y. enterocolitica is heat labile. No survivors were detected in beef roasts inoculated with 10^6 cells/g, which were heated to an internal temperature of 60°C. In another study by Hanna, et al. (100), 10^6 to 10^7 Y. enterocolitica/ml skim milk did not survive heating to 60°C for 1-3 min. Similar results were obtained by Francis, et al. (81). The authors found that Y. enterocolitica is not heat resistant and is destroyed by standard pasteurization times and temperatures. However, post-pasteurization contamination is possible and Y. enterocolitica can multiply to infectious levels in milk-holding vats if sanitation is not done properly. Hughes (109) was able to isolate Y. enterocolitica from pasteurized milk vats at two factories. The sanitation protocol at factory A was modified to include a steam cleaning process. Since that time no isolations of Y. enterocolitica have been made. A rubber collar on the holding vat was implicated as the source of contamination at factory B.

Yersinia cannot survive the 60 d aging period required for Cheddar cheese manufactured from raw milk. Schiemann (194) isolated Y. enterocolitica from 18.2% of raw milk samples from vats or holding tanks at cheese manufacturing plants. The isolation rate for cheese curd was approximately half, 9.2%. This shows that the cheese manufacturing process itself is not favorable for Y.

enterocolitica survival. Only one of these curd samples was still positive after 4 weeks of storage at 4°C, and this sample was negative by week 8. Moustafa, et al. (167) showed that Y. enterocolitica strain E675 was present at approximately 200cells/g in a Colby-like cheese after 8 weeks of storage at 3°C.

IV. Yersiniosis

A. Gastrointestinal Infections

Y. enterocolitica has been associated with various human illnesses such as enteritis, terminal ileitis, and mesenteric lymphadenitis. Common clinical symptoms include fever, abdominal pain, and diarrhea. Nausea and vomiting rarely occur. The symptoms are more severe in children. The illness usually appears in 24-36 h after ingestion of the cells and is self-limiting, lasting 1-3 d. Long-term complaints have been reported.

The ileocecal portion of the digestive tract is most commonly affected. The primary lesion is a result of invasion of the wall of the small intestine. Ulceration of the intestinal mucosa at the site of the lymphoid tissue may result in an extensive loss of blood and fluid (116). Enlarged medenteric nodes produce severe abdominal pain and may result in surgery for appendicitis (198). An uncomplicated case of yersiniosis resembles salmonellosis or shigellosis. Occasionally, serious complications occur. Leino described a case a mesenteric

lymphadenitis that resulted in superior mesenteric vein thrombosis and gangrene of the small intestine (198). Peritonitis has been reported but occurs only when some other pre-existing illness is present.

B. Extraintestinal Infections

Septicemia - Once thought to be rare occurrence in association with Y. enterocolitica infection, many cases of septicemia have been reported. Early reports proposed that septicemia occurred only in elderly or immunocompromised patients. Cirrhosis, diabetes, and pneumonia are suspected to be predisposing conditions. Reports of septicemia in healthy infants demonstrated that it was not limited to the elderly with compromising illnesses.

Arthritis - Antecedent yersiniosis has been identified as a cause of reactive arthritis and Reiter's syndrome. These complications are more likely to develop in patients $\leq$ 10 years old. Leirisalo discovered that the areas most often affected were the lower extremities (94%), upper extremities (65%), and the back (29%) (198). The symptoms usually last 12 -396 months with an average of 78 months. Most of the cases of arthritis are found in European and Scandinavian countries, where serovars O3 and O9 predominate in human infections.

Erythema Nodosum - Erythema nodosum is a cutaneous granuloma that has been linked with tuberculosis. In recent years, it has been associated with several other infections, but rarely with intestinal infections.

Miscellaneous - Sarcoidosis, skin infections, eye infections, endocarditis, thyroid disorders, glomerulonephritis, liver disease, respiratory infections, neurologic complications, immunoblastic lymphadenopathy, muscle abscess, osteomyletis, cervical adenitis.

V. Antimicrobial Susceptibility

Y. enterocolitica is usually susceptible to the aminoglycosides (gentamicin, kanamycin, streptomycin), polymyxin, chloramphenicol, trimethoprimsulfamethoxazole, and newer beta-lactam antibiotics, especially ceftriaxone, cefotaxime, ceftizxime, and cefmenoxime (198). Studies by Scribner, et al. (202) demonstrated the inhibition of 90% of isolates by ceftizoxime and ceftriaxone at a concentration of 0.06 μg/ml. Hornstein, et al. (107) reported similar results with ceftriaxone, cefotaxime, ceftizoxime, and cefmenoxime (0.06-0.08 μg/ml). In addition, these authors found that all of the Y. enterocolitica isolates had some degree of resistance to ampicillin and cephalothin. Vidon and Delmas (226) reported resistance to ampicillin and sensitivity to streptomycin, chloramphenicol, tetracycline, sulfonamides, and mercuric ions. The organism is resistant to penicillin and many penicillin derivatives, with the exception of mecillinam and some cephalosporins.

VI. Epidemiology

Food was quickly recognized as an important source of infection. Reports of yersiniosis in the United States have suggested that food is the vehicle of transmission. Four of the five documented outbreaks in the U.S. have involved serovar O8, which appears to be restricted to North America.

A. Milk

The largest foodborne outbreak of yersiniosis in the United States involved pasteurized milk, which is usually considered a safe product. The implicated serovar (O13) was previously only recognized in monkeys (198). This 1982 outbreak was estimated to affect several thousand people. Adult patients developed pharyngitis with positive throat cultures, symptoms not usually associated with yersiniosis. The epidemiology of the outbreak was very unusual. Pigs were implicated as the source but never found to be carriers of the same serovar. Milk crates used to transport outdated milk were contaminated by mud at the pig farm. These same crates were superficially washed and used to transport freshly pasteurized to the market. The bacteria remained on the outside of the carton and were presumably introduced into the product after opening by the consumer. Y. enterocolitica was able to grow to infectious levels under refrigerated storage. Stanfield (212) showed that Y. enterocolitica could survive for at least 21 d on the outside of the milk cartons stored at 4°C. In 1976, an

outbreak occurred affecting 218 schoolchildren. Thirty-three children were hospitalized and 13 had unnecessary appendectomies. Y. enterocolitica O8 was isolated from the chocolate milk. In a study from France, Vidon and Delmas (226) found that 69.5% of raw milk samples taken from January to March 1980 were positive for Y. enterocolitica. In a second survey in April of the same year, the authors found that 92.3% of the samples were positive. The results show a higher occurrence of Y. enterocolitica in the spring rather than in the winter. Sporadically, Y. enterocolitica is isolated from pasteurized milk. This is probably due to post-pasteurization contamination and not survival of the organisms during processing.

B. Water

An early report of yersiniosis in the United States was linked to the consumption of water from a mountain stream. There have been a few reports of yersiniosis that suggested that water was the source of infection. Water may have been the source of a infection of skiers at a Montana resort (198). Christensen (31) states that Y. enterocolitica was isolated from 24% of water samples taken from sources near dairy farms. This indicates the importance of water as a vehicle of infection to humans and animals directly or indirectly via raw milk or other food products. Water used in tofu manufacturing was implicated as the source of Y. enterocolitica serovar O8 infection. From December 1981 - February 1982, 87

cases of yersiniosis were reported in Seattle, WA (10). The patients ranged in age from 2 months to 74 yrs. According to Tacket (221), the most common clinical symptoms were gastrointestinal infections such as abdominal cramps, fever, headache, diarrhea, and photosensitivity. Two patients had appendectomies and one had a partial colectomy. Aulisio (10) determined that two antigenically distinct strains were involved, O8 and O:Tacoma. At the tofu manufacturing plant, serotype O8 was recovered from water samples. Although person-to-person contact is considered a vehicle for transmission, no secondary outbreaks occurred among family members who did not eat the tofu. Untreated water may be a potential source of contamination because Y. enterocolitica has simple nutritional requirements and grows well at low temperatures.

C. Swine

Pathogenic strains of Y. enterocolitica are commonly isolated from healthy pigs at slaughter. Pigs appear to carry Y. enterocolitica in their normal throat flora. Young pigs become carriers within 1-3 weeks after exposure, and remain colonized for long periods of time without reinfection. Newborn pigs are easily colonized and become intestinal and pharyngeal carriers. A survey by Christensen (32) found that an average of 26% of healthy pigs carried pathogenic Y. enterocolitica in their tonsils. This study showed a herd-wide distribution, with some herds testing almost 100% positive. The contaminated herds were of the

"open management" type. Six to eight week old pigs were bought from markets. The pathogen-free herds were closed and herd growth was only due to births on the farm. These closed farms had an infection rate of $\geq$ 1%.

It seems reasonable to assume that since Y. enterocolitica is so prevalent in pigs it would be spread to the consumer through pork products much like Salmonella in poultry products. Yet, only fresh porcine tongues consistently shown the presence of pathogenic Y. enterocolitica, usually serovar O3. This does not mean that other pork products are Y. enterocolitica-free, instead it could demonstrate the inability of enrichment and isolation procedures to detect low numbers of cells in a mixed population. With the exception of fresh pork tongues, no other food reservoir of Y. enterocolitica has been identified.

D. Person-to-Person Transmission

Reconstituted powdered milk and/or chow mein were implicated as the source of infection in a 1981 outbreak at a summer diet camp. Information obtained by Morse, et al. (161) suggests that a food handler was the source of Y. enterocolitica O8. Fifty-three percent of the campers and staff complained of abdominal pain, fever, diarrhea, and/or nausea and vomiting. These researchers found that the head cook had no symptoms, but had positive Yersinia stool cultures.

Person-to-person transmission was suspected as a route of infection of family

members of two nurses who complained of severe abdominal pains, diarrhea, and fever (198). Family members displayed the same symptoms. Schiemann (198) cites another case in which nine hospitalized patients, seven elderly women and one premature neonate developed symptoms os yersiniosis. Serovar O5 was isolated from the patients. This serovar normally exhibits no pathogenicity nor do they possess any of the recognized virulence factors. All of the patients had other illnesses or were receiving treatments such as radiation. This outbreak demonstrated that under certain conditions serovars of Y. enterocolitica that cannot be identified in the laboratory as pathogens may cause disease.

VII. Enrichment

Several selective enrichment media have been developed, but only modified Rappaport and bile-oxalate-sorbose (BOS) broths have received much attention or appear to hold any great promise. Modified Rappaport broth is inhibitory to several strains. Carbenicillin was used in the original formulation but subsequent studies have found that its presence is not required. Since it can be inhibitory to some strains of Y. enterocolitica, it has been eliminated from the formula. Schiemann (196) shows that enrichment in BOS broth is an improved method for recovery of a large number of Y. enterocolitica strains from inoculated as well as natural foods in 3-5 d at 25°C. Walker and Gilmour (227) found that a BOS broth enrichment followed by enumeration on CIN agar was superior among 26

enrichment procedures for the recovery of Yersinia from milk. Aulisio, et al. (9) described an alkali method for rapid recovery of Y. enterocolitica from foods. The authors showed that treatment with dilute potassium hydroxide increased the yield of Yersinia species fourfold and improve the sensitivity 100 X. Also, this treatment shortened the incubation time and suppressed the growth of non-Yersinia species. However, Schiemann (197) studied the alkalotolerance of Y. enterocolitica and found that alkali treatment was not highly successful in comparison with BOS broth. He stated that the variable conditions associated with food enrichment prevents standardization of the procedure. Fukushima (86) found that a dilute alkali treatment proved to be a valuable and time-saving alternative for direct isolation of Yersinia from meat samples contaminated with approximately 10^2 cells/g. Doyle and Hugdahl (53) report similar results with the KOH-postenrichment proving excellent recovery of Y. enterocolitica in 1-3 d.

VIII. Plating Media

The earliest plating media for isolating Y. enterocolitica were selected from available enteric agars such as salmonella-shigella (SS), xylose-lysine-deoxycholate (XLD), deoxycholate-citrate-lactose (DCL), MacConkey, Hektoen, bismuth sulfite, and lactose-saccharose-urea (LSU). Incubation at 22-28°C for 48 h is used because selective agents in the media can become toxic at higher temperatures.

MacConkey agar has been a popular isolation media for Y. enterocolitica. Y. enterocolitica colonies appear colorless to slightly pink (nonlactose fermenter) on MacConkey agar incubated at 22-25°C for 48 h. Colonies appear small, slightly raised, with a smooth surface and entire edge.

Attempts have been made to modify these media to improve their selectivity for Y. enterocolitica. Salmonella-shigella (SS) agar supplemented with sodium deoxycholate has increased sensitivity. In a study of growth characteristics and response to selective agents, Schiemann (195) demonstrated that Y. enterocolitica had a high tolerance for surface active agents. However, cetrimide, 4-nitrophenol, potassium tellurite, and sodium azide were inhibitory at low concentration. Also, he reported a resistance to Irgasan at levels inhibitory to other Enterobacteriaceae. In the same report, minimum inhibitory concentration (MIC) tests of 57 antibacterial agents suggest that carbenicillin, novobiocin, and SCE-129 (cefsulodin) might be useful in selective isolation of Y. enterocolitica. Cellobiose-arginine-lysine (CAL) and cefsulodin-irgasan-novobiocin (CIN) agar are commercially available selective media. Walker and Gilmour (227) evaluated nine selective plating media and found CIN agar to be superior for isolating Y. enterocolitica from milk samples. The authors reveal that pre-enrichment in trypticase-soy broth (TSB) (24 h @ 22°C) followed by selective enrichment in BOS broth (5 d @ 22°C) and plating on CIN agar (48 h @ 25°C) allowed the greatest increase in the numbers of Yersinia spp. and maximum inhibition of non-

Yersinia species. CIN agar is highly selective for Y. enterocolitica and good growth is evident within 18-20 h at 32°C. Colonies on CIN agar are 0.5-1.0 mm in diameter, slightly raised, smooth with an entire edge, and with a distinctive red "bullseye" with a transparent border.

IX. Identification

Kligler iron agar (KIA) and Christensen's urea agar, both incubated at 35°C are biochemical tests used to identify Yersinia species. KIA is preferred over triple-sugar-iron (TSI) agar, which contains sucrose. Y. enterocolitica gives a reaction on TSI identical with lactose fermenters (acid/acid). Urease-negative strains have been isolated, but there is no firm evidence that any of these strains are pathogenic to humans. Identification of Y. enterocolitica requires the following biochemical tests (incubated at 25°C): sucrose (+), rhamnose (-), raffinose (-), melibiose (-), alphamethylglucoside (-), and Simmon's citrate (-). Weagant (230) developed the lysine-arginine-iron agar (LAIA) for the presumptive identification of Y. enterocolitica. The LAIA incorporates five biochemical reactions into a single tube medium. This medium was developed to simplify the identification of Y. enterocolitica from samples of fresh and marine water, shellfish, and marine sediment. Reactions were found to be reliable and distinctive, allowing non-Yersinia species to be eliminated.

X. Virulence Factors

According to Joklik (116), the major virulence factors for Y. enterocolitica include:

1. V and W antigens and an Lcr similar to Y. pestis
2. Adherence to human epithelial cells in tissue culture
3. A positive guinea pig corneal ulcer test (the Sereny Test)
4. Heat-stable enterotoxin production
5. Pestin sensitivity
6. Autoagglutination and surface fibril production
7. An outer membrane protein that is associated with increased resistance to the bactericidal activity of human serum, hydrophobicity, and a surface change in the bacterial surface charge.

The V antigen is a 38Kdal cytoplasmic protein, and the W antigen is a membrane-associated or extracellular 145 Kdal lipoprotein. These antigens are synthesized at 37°C under calcium-deficient conditions by plasmid-positive Yersinia. The V antigen appears to be a major virulence factor in Yersinia. The V and W antigens are important for providing protection for Y. enterocolitica and allowing it to grow, rather than be killed, after phagocytosis by macrophages (52).

XI. Injury

Few studies have been conducted on the repair and recovery of injured Y. enterocolitica. Injured cells have an increased sensitivity to sodium chloride and sodium deoxycholate. Ray (183) states that preliminary incubation of injured cells in a nonselective media followed by addition of deoxycholate and bile salts will allow for the recovery of noninjured and repaired Y. enterocolitica. El-Zawahry and Grecz (62) show that with the same amount of cell inactivation, freezing caused substantially greater cell injury than radiation. Also, they indicate that 100-150 krad would be sufficient to inactivate 10^{10} Y. enterocolitica if irradiated at 2 and -18°C, respectively. In the same study, the authors showed that the addition of 2.5% sodium chloride will result in a 50% reduction of the required dose to achieve sterility. Studies by Singh, et al. (203) and LeChevallier, et al. (128) demonstrated a reduced virulence of injured Y. enterocolitica. According to Singh (203), copper (0.75 mg/L) caused substantial injury (87 to 95%) of Y. enterocolitica serovar O8 in 72 h at 4°C. Copper-injured cells had a higher lethal dose in mice (2,700 CFU) than uninjured cells (150 CFU). LeChevallier, et al. (128) showed that injured Y. enterocolitica were 20 times less virulent when exposed to chlorine than control cells. In addition, they stated that higher doses of chlorine, 0.9-1.5 mg/L, were necessary to injure Y. enterocolitica compared with 0.25 to 0.5 mg/L for enterotoxigenic E. coli or coliform bacteria.

IMPEDANCE MICROBIOLOGY

I. Background

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) (72). A decrease in electrical resistance of a medium occurs when actively growing cells utilize large molecules resulting in smaller ion pairs (99). 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. (28) stated that these chemical changes alter the electrical resistance or impedance. Impedance can be described as a measure of the total opposition to the flow of an alternating electrical current in a circuit. Any change in the components of the circuit, including bacteria and their metabolic products, alters the impedance, and influences the voltage-current relationship (99). Several physical and chemical factors may also affect impedance. These include temperature fluctuations, evaporation, absorption of gases, and degradation of the culture medium during incubation. In addition, the nature of the electrodes, culture medium used, and growth charateristics of the organism can greatly influence the impedance (159). 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.

As early as 1899, Stewart (214) described changes in electrical conductivity of putrifying blood. In similar experiments in 1912, Oker-Blom found a tenfold change in conductivity over a 25 h period (99). In 1926, Parson and Sturges (175) noticed a direct proportionality between conductivity and ammonia production in the case of putrefactive anaerobes. Twelve years later, Allison (4) proposed that changes in conductivity correlated with enzymatic hydrolysis of proteins. Firstenberg-Eden (69) 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. Impedance microbiology is a rapid, reliable and easy method of enumeration.

II. Applications

A. Blood Culture Testing

Bacteremia can be life threatening if not treated quickly with the appropriate antibiotic. In conventional blood culture processing, much time is spent doing smears and subculturing samples that ultimately prove negative. A rapid detection method such as impedance speeds this process so that effective

treatment may begin. Specter, et al. (206) used inoculated blood cultures and found impedance testing to be highly advantagous. They detected growth of 10 species of Gram positive and Gram negative bacteria in 6-18 h. Buckland, et al. (24) detected 10^2 and 10^6 cfu/ml in 6 and 2 h, respectively. Compared to conventional blood culture methods, results were obtained 12 h earlier. Hadley, et al. (98) developed a rapid blood screening technique which had a sensitivity of 93.6% and specificity of 98.0%. Tests by Kahn, et al. (119) showed that the time for blood culture testing could be reduced from 24 h to 8.5 h.

B. Urinalysis

Examination of urine cultures is the most frequent test done by clinical laboratories. A significant portion of these cultures will be negative, therefore a rapid screening test would be useful to select for those samples that require further work. Throm, et al. (223) found that a 2-3 h incubation was sufficient for the detection of 41 clinically significant bacteria. Zafari and Martin (234) and Cady, et al. (27) examined urine cultures with 10^5 cfu/ml and found a 2 and 2.6 h incubation, respectively, to be the most effective screening time. They also found that an increase in incubation time reduced false negatives but increased false positives.

C. Food Product Testing

Microbiological testing of food products allows for an evaluation of food quality and wholesomeness. Conventional techniques require colony formation. This type of testing is labor intensive, time consuming, and retrospective. Rapid methods reduce storage space and labor needs, and allow product to move to market more rapidly (68).

Impedance microbiology 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 (70). Martins and Selby (146) developed an impedimetric MPN technique to rapidly estimate coliforms in meat. Impedance has also been used to examine raw milk samples (68, 71, 90). Firstenberg-Eden and Tricarico (71) 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 (68). Gnan and Luedecke (90) compared raw milk SPC to IDT and found that 97% of the milk samples could be classified correctly within 7 h. They also found that impedance results for raw milk, raw milk + yeast extract (YE), raw milk + preliminary incubation (PI, 18 h @ 13°C), and raw milk + PI + YE correlated with the SPC with coefficients of -0.77, -0.88, -0.78, and -0.79, respectively.

Attempts have been made to accurately determine the potential shelf-life of dairy products with the use of impedance (15, 16, 118, 147). Bishop, et al. (16) 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 h. Similar results were reported by Martins, et al. (147). They found that impedance measurements provided a better means of estimating shelf-life than the SPC or psychrotrophic plate count (PPC).

Gaspar-Rolle (88) and Mosteller and Bishop (164) used impedance microbiology to estimate the nmbers of bacteria attached to food processing surfaces. Gaspar-Rolle (88) found that Pseudomonas fragi, Bacillus cereus, and Listeria monocytogenes could attach to gasket materials after 2, 12, and 24 h of bacteria growth, respectively. Mosteller and Bishop (164) examined sanitizer efficacy towards attached bacteria using impedance microbiology. Buna-N rubber and teflon gasket pieces were placed directly into module wells to determine the number of survivors after sanitizer treatment.

In addition to these uses, impedance microbiology has been used for the detection of post-pasteurization contamination of cream and for niacin assays (60, 95). Sorrells (205) demonstrated that impedance results were equivalent to the usual plate count method for grains but were obtained more quickly.

Impedance techniques have also proved useful for microbial analysis of frozen vegetables (102), and frozen orange juice concentrate (231). Detection of bacterial contamination in the brewing industry requires the development of a visually detectable haze which may take 1-2 weeks. Evans (63) showed that impedance responses could give an accurate indication of beer spoilage capacity in 2-4 d. Impedance has been used for the detection of *Salmonella* in confectionary products (7), milk chocolate (25), and animal feeds (204).

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

All product contact surfaces must remain clean in order to prevent contamination of the final food product. Dunsmore, et al. (57) defined a "cleaning system" as those practices that maintain the product-contact surfaces in a condition that ensures that they do not impair quality of the final food product. All cleaning systems are based on three parameters (57). First, the system must remove any food residues that can serve as a source of nutrients for remaining microorganisms. Also, the cleaning system must destroy any cells that were not physically removed with food residues. Finally, equipment must be stored under conditions that discourage 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 complementary processes; neither can effectively achieve the desired results alone. Dunsmore (55) suggested that the detergent is the most important system component for controlling bacterial numbers, with the sanitizer exhibiting some control and rinses very little. Forwalter (79) stated that effective cleaning of equipment is 90% of the total sanitizing job. Although cleaning reduces bacterial numbers on surfaces,

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

Concentration, temperature, exposure time, and external energy determine the effectiveness of chemical cleaners (140). Increasing the concentration will enhance effectiveness, however, this is probably the least important factor. An increase in 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 will be more effective with 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 the cleaning solution 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 (140).

Harper (104) described cleaning in terms of a series of five basic steps. First, prerinsing serves to reduce the soil load. Next, the detergent solution is applied to penetrate the soil. Soil is displaced by saponifying the fat, peptizing the proteins, and dissolving the minerals. Dispersion, defloculation, and emulsification suspend soil in the solvent. Finally, redeposition is prevented by providing a final rinsing step.

Bacteria are controlled by cleaning or physical removal and by 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 will determine the effectiveness of sanitizers. Sanitizer performance depends on the substrate, soil, formulation, application method, and the cleaning system in which it is applied (57). When examining sanitizer efficacy, the number of surviving organisms, and not the number killed, is the significant factor (8). Sanitizers commonly used in the dairy industry include: chlorine, iodophor, acid anionic, peroxyacetic acid, fatty acid, and quaternary ammonium compounds.

I. Chlorine

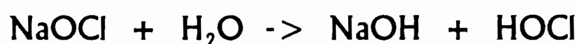
In 1881, Koch demonstrated in the laboratory, under controlled conditions, the lethal effects of hypochlorites on pure cultures. The first large scale use of chlorine was for the purification of water in Chicago in 1908. Widespread acceptance of chlorine as a disinfectant came during World War I when Dakin (47) introduced a 0.50% sodium hypochlorite solution for treating wounds. Since then, 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 (110). Chlorine related compounds can be subdivided into five categories: 1) liquid chlorine, 2) hypochlorites, 3) inorganic chloramines, 4) organic chloramines, and 5) chlorine dioxide. Hypochlorites

(HOCl) are the most active form of chlorine sanitizer, and they are probably the oldest and most widely used form. Hypochlorites are (a) proven and powerful germicides that can destroy a broad spectrum of microorganisms, (b) deodorizers, (c) nonpoisonous to humans at in-use concentrations, (d) colorless and nonstaining, (e) easy to handle and (f) economical (58).

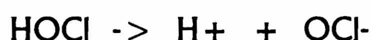
Hypochlorites have some limitations such as a short shelf-life, a penetrating and irritating odor, corrosiveness to some metals, and are adversely affected by organic matter (58, 87, 224). pH can greatly affect biocidal activity. An increase in pH to alkaline values causes a substanial decrease in effectiveness. A 1941 study at Iowa State College demonstrated the effect of pH on 25 ppm chlorine. At pH 6, 2.5 minutes were required for a 99% reduction in a suspension of Bacillus metiens. At pH 7, 8, 9, 10, and 12.86, the time necessary for the same 99% reduction of B. metiens was 3.6, 5, 19.5, 131, and 465 minutes, respectively. Results indicated that these dramatic differences in biocidal activity are due to changes in the concentration of undissociated hypochlorous acid (58). The microbial activity of hypochlorites is dependent upon HOCl concentration. Studies by Costigan (39) showed the effects of increased temperature on the destruction of Mycobacterium tuberculosis using 50 ppm hypochlorite solution at pH 8.35. Complete destruction was observed in 30 s at 60°C. When the temperature was decreased to 55°C, the cells were destroyed in 60 s. At 50°C, 2.5 minutes were required for complete destruction.

Several theories have been proposed to explain the method of microbial inactivation by chlorine. The antimicrobial action of chlorine is due in part to the formation of toxic N-chloro compounds. These compounds interfere with cell metabolism, inhibit glucose oxidation, and/or oxidize sulfhydryl groups (84, 85, 140). Other experiments demonstrate that chlorine will interfere with the transportation of extracellular nutrients by impairing membrane functions. Chlorine has also been shown to be effective against spores (125, 233). Kulikovsky, et al. (125) found that spores exposed to chlorine lose Ca^{++}, RNA, and DNA due to altered permeability.

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



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

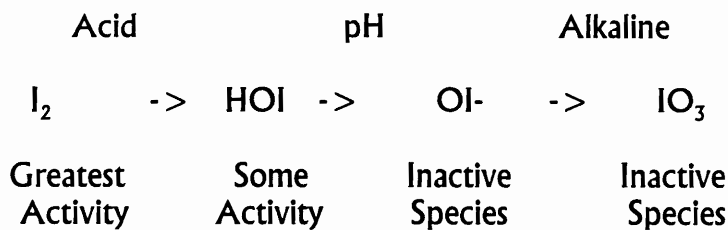


$$HOCl \rightarrow H^+ + OCl^-$$

The dissociation of hypochlorous acid to hypochlorite ion is greatly influenced by such factors as pH, temperature, and ionic strength (160). 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.

II. Iodophor

Like chlorine, iodine has been useful in water treatment and for sanitation of processing equipment and surfaces. Elemental iodine is highly reactive and is thought to be the active form of iodine sanitizers (173). The effect of pH on the form of sanitizer present is shown below:



Iodine is destructive toward a complete spectrum of microorganisms. Iodine and chlorine are equally effective against vegetative cells (93, 105, 139, 162). Iodine is less effective in inactivating spores (40). Compared to vegetative cells, spores are ten to one thousand times more resistant. The lethal action is rapid and acquired resistance to iodine does not occur. Except at low concentrations, iodine's 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. Iodine will stain clothing, skin, and other materials. In addition, it can be a harsh irritant to the skin and mucous membranes. Aqueous solutions and tinctures evaporate quickly leaving iodine crystals on the epithelial

cells causing irritation (33).

In 1949, Shelanski found that certain surface-active agents such as polyvinyl pyrrolidone could solubilize iodine to form a complex that 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 (224). 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 (215).

The exact destructive mechanism of iodine has not been fully explained but may be assumed that iodine interferes with protein function (92). Iodine reacts with basic N-H bonds of some amino acids and bases of nucleotides which in turn prevents hydrogen bonding and causes a loss of protein function. Iodine also may oxidize the S-H group of cysteine causing disruption of disulfide bridges that can be an important factor in tertiary structure of proteins. 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 skin, equipment, utensils, and work surfaces. A strongly acid iodophor can be used to prevent and remove milkstone deposits from dairy equipment (87). This is possible because the optimum pH for use is between 3-4.

Iodine gives milkstone a yellow tint which aids in visualization of cleaning. The surfactants that accompany iodine in the iodophor complex help to remove some soils left on equipment surfaces.

III. Acid Anionic

Acid anionic sanitizers are gaining acceptance because they possess the following advantages: rapid, effective against a broad range of microorganisms, noncorrosive and nonstaining, no objectionable odor, effective in removal and prevention of milkstone, and a high stability. The main disadvantages with this sanitizer are: slow action against spores, foaming, and use at acid pH. Acid anionic sanitizers have been used in the dairy industry for the past 30 years with good success. Researchers have found that an acid anionic sanitizer will effectively eliminate bacteria associated with mastitis. Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, S. aureus, and Pseudomonas aeruginosa can be effectively destroyed in 30 s (59).

Acid anionic sanitizers are a combination of an 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 (18). Because of the acidic 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. These compounds are composed of two principal functional groups, lipophilic and hydrophilic. 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 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 (50).

Acid anionic sanitizers destroy bacteria by the general denaturation of cellular proteins. Disruption of cell membranes and permeability barriers are proposed mechanisms of inactivation (12, 172, 186). Baker, et al. (12) described rapid disorganization of cell membranes that were treated with anionic surfactants. In addition, enzymes essential to cell metabolism are inactivated (59). Acid anionic compounds are effective against a wide range of microorganisms, including thermodurics, bacteriophages, and most yeast strains (79, 134). Other advantages include the lack of an objectionable odor and production of a residual anti-bacterial film. They are effective in the presence of organic matter and in hard water. These sanitizers must be used at an acid pH, which can be corrosive to some metals. Acid anionic sanitizers are not effective in the destruction of most spores.

IV. 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. Peroxyacetic acid is a stabilized equilibrium mixture of hydrogen peroxide, peroxyacetic acid, and acetic acid. This stabilized mixture has gained widespread acceptance because it eliminates many problems. Peroxyacetic acid by itself can be corrosive on some metals and has a characteristic pungent odor. In comparison to hydrogen peroxide, this sanitizer is much more effective against a broad range of microorganisms at lower temperatures and concentrations (13, 201, 225). In 1902, Freer and Novy (83) first reported that peroxyacetic acid was effective in destroying vegetative cells and in the mid-1960's, Spoessing and Muecke (208, 209, 210) made similar observations, but because of stability and handling problems, acceptability was low.

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 (187). The same study indicated that $\leq$ 50 ppm of the sanitizer does not cause turbidity in beer, and does not adversely affect foam height, color, taste, odor, or pH.

Studies by the Danish Government Research Institute determined that up to

10 ppm peroxyacetic acid did not adversely affect the color, pH, odor, and taste of milk (187). Peroxyacetic acid is less corrosive than other sanitizers such as iodophor, acid anionic, and chlorine and is 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 (13, 19, 111, 112, 123, 187). Hutchings and Xezones (111) tested 23 antimicrobials and found that peroxyacetic acid was the most effective. Greenspan and MacKellar (94) concluded that peroxyacetic acid was more effective than hydrogen peroxide and acetic acid. The authors demonstrated bactericidal effects at 0.001%, fungicidal effects at 0.003%, and sporicidal properties at 0.3%. The primary mechanism of action is irreversible oxidative damage to cellular components (225). Recent tests suggested that this compound is effective against L. monocytogenes strains and bacteriophages (130, 200).

V. Fatty Acid Sanitizer

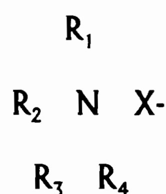
The active ingredients in the fatty acid sanitizer are phosphoric and citric acid. In addition, octanoic and decanoic acids are present as inert ingredients. Like acid anionic sanitizers, this sanitizer is effective against a broad range of microorganisms. Continual use is also effective in the prevention of mineralstone

formation on processing equipment. Studies show that this sanitizer is effective against Salmonella and Listeria (122). Fatty acid sanitizers are effective on food contact surfaces as well as noncontact surfaces. They are noncorrosive to stainless steel equipment and can be used during an acid rinse cycle which lessens effluent discharge during clean-up. These sanitizers have the disadvantage of a fatty acid odor, but they are effective at a low pH and has low foaming properties.

VI. Quaternary Ammonium Compounds

The quaternary ammonium compounds or QUATs are surface active disinfectants with both hydrophilic and hydrophobic groups. They are commonly referred to as "cationic detergents", although the detergent action is diminished at the concentration used for disinfection (33, 87). 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. Few free molecules are found in solution; however, this means that QUATs cannot be easily rinsed off surfaces. QUATs are the result of nucleophilic substitution of alkyl halides with tertiary amines. 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:



where the X- is usually a halogen and R_1, R_2, R_3, and R_4 are alkyl groups. At least one R group is a long hydrocarbon chain derived from fatty acids with 8-18 carbon atoms. The remaining R alkyl groups may be alike or different, substituted or unsubstituted, saturated or unsaturated, branched or unbranched, and they may contain ether, ester, or amide linkages (178). The hydrophobic chain allows the molecules to congregate at interfaces. The nitrogen atom plus the four alkyl groups form a positively charged cation, which serves as the functional portion of the molecule. The anion portion exhibits no antimicrobial activity (33, 87, 173). The cation has a high affinity for water and prevents separation at the aqueous phase (87). 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 (178). They are only slightly toxic or irritating to human tissues. QUATs do not decompose at high temperatures and radiation sterilization. Poor rinsibility of QUATs can lead to undesirable traces in foods and throughout a processing plant. Useful applications of QUATS include dairy farms and dairy plants; egg processing plants; the fishing, brewing, and sugar refining

industry.

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 at this concentration. QUATs are not effective against viruses, although lipophilic viruses are susceptible to some QUATs. Since QUATS are cationic surface agents, they reduce surface tension at interfaces, and are attracted by and absorbed onto negatively charged surfaces such as wool, glass, proteins, and bacteria at alkaline pH. Ionic micelles form, which alters electrical conductivity, surface tension, and solubility. The surface-active properties can cause precipitation, flocculation, and denaturation of proteins (178). The leading explanations for the mechanisms of inactivation are protein denaturation and enzyme disruption (181); interference with aerobic and anaerobic respiration (12); cytolytic damage and phosphorus loss (6); membrane damage and potassium loss (193).

QUATs are most effective at a pH around 10. pH values in the neutral range are unfavorable. Their activity is enhanced by increasing the temperature of the solution. Like hypochlorites, 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 that carry the opposite electrical charge may also inactivate QUATs.

MECHANISMS OF ADHESION

Physicochemical forces are involved in the attraction or electrostatic repulsion of bacteria to or from surfaces. Bacteria are transported to the surface by gravitational forces and Brownian motion. Long and short range forces hold the bacteria near the surface, while firm attachment occurs by means of cellular appendages such as pili or exopolysaccharides. Any study of attachment mechanisms must consider the bacterial surface, substratum, surrounding medium, and other influencing environmental factors.

Marshall (142) refered to bacteria as living colloid particles, ranging from about 0.2 μm to several micrometers in length, with most bacteria measuring 1.0 μm in length or diameter. At the pH values of most natural environments, bacteria possess a net negative surface charge. This net negative charge can be reversed at low pH values, which indicates the presence of some charged basic amino groups, which are evident when acidic, carboxyl and phosphate groups remain undissociated. The overall surface free energy of cells will vary, with some bacteria being hydrophilic and others relatively hydrophobic. Hydrophobic regions of bacterial cells may not be uniformly distributed and could affect the orientation of bacteria at interfaces.

Costerton (36) stated that bacteria are not inert particles, but are living colloidal suspensions that exhibit growth and metabolism. The metabolism of

attached bacteria is evident in the production of extracellular polymers. These exopolymers, in association with flagella, pili, and fimbriae are essential elements in the adhesion process. Production of extracellular polymers varies with bacterial species, age, and growth conditions. These polymers can have an effect on the quantity and tenacity of adhesion occurring in natural ecosystems.

Once bacteria contact a surface, the attachment process begins. Marshall (142) described a two-step attachment process, with a reversible and an irreversible phase. Primary or reversible binding holds bacteria close to the surface by weak attractions, such as surface charges, van der Waal forces, and hydrophobic interactions. Secondary or irreversible binding occurs when physical and chemical forces combine to hold the bacterial cells firmly to the surface. These forces include the production of proteinaceous or polysaccharide adhesions and the formation of a biofilm matrix.

Colonization of surfaces depends on the bacterial species present. When several bacterial species are present, colonization patterns change because each species reacts differently to the surface. One type of bacterium may adhere, preventing the adherence of other species or one species may act as a primary colonizer and facilitate the attachment of other types of microorganisms (38).

I. Transport of Bacteria to Surfaces

Bryers and Characklis (23) noted that the rate of overall bacterial adhesion

to surfaces in turbulent flow conditions increases with biomass concentration. In other words, aqueous environments with high concentrations of bacteria have a high incidence of adhesion. Consequently, in dilute bacterial suspensions, transport to the surface is probably the rate-limiting step in bacterial deposition. Under conditions of turbulent flow, a zone of relatively still liquid exists near a solid surface. This viscous (or boundary) sublayer is dependent on the amount of fluid shear rate present in the system. Bacteria in the aqueous phase must be able to penetrate this viscous sublayer to be transported to the solid surface (142).

Sedimentation is relatively important in systems with low-shear and large particles, such as large bacteria or bacterial aggregates. Most bacteria exist in stable suspensions and sedimentation occurs only when the system becomes destabilized as in floc formation. In the turbulent flow systems found in most natural habitats, sedimentation of bacteria is unlikely to occur (142).

Many bacteria are capable of independent motion by means of flagella. Bacterial motility increases the number of opportunities for a bacterium to approach a surface. The motion of the flagella cannot, however, overcome the repulsion barrier near the surface. Motile bacteria are capable of exhibiting chemotactic responses in the presence of certain nutrient sources. These responses allow a bacterium to take advantage of the nutrient concentration gradient that exists at the solid-water interface. Under certain circumstances, bacteria actively move toward a surface and may be held near the substratum by certain attractive

forces. On the other hand, bacteria exhibit negative chemotactic responses when inhibitory substances are present. Chemotactic responses may transport cells through the viscous sublayer, but are probably not significant under turbulent flow conditions (142).

The magnitude of displacement of particles by Brownian motion depends upon particle size (142). The particle size of most bacteria exhibiting Brownian motion is less than 1.0 μm. Bacteria with a particle size > 1.0 μm do not display this type of motion. Brownian motion probably has little effect on the transport of bacteria under turbulent flow conditions, but may be a important factor within the viscous sublayer.

The degree of cell surface hydrophobicity depends on bacterial species. Hydrophobic bacteria are repulsed from the aqueous phase and attracted to solid surfaces. Ultimately, cell surface hydrophobicity could provide a mechanism of transport between bacteria and the substratum. In addition, the irregular distribution of hydrophobic sites could provide a means for a particular orientation of some bacteria at the solid-surface interface.

Bacteria under turbulent flow conditions are dispersed by eddy diffusion in the turbulent core region, thus maintaining a uniform concentration in the bulk liquid phase (142). Eddy diffusion transports the bacteria to the viscous sublayer (120). Once in the viscous sublayer, Marshall (142) noted that the bacteria encounter significant frictional drag forces, which gradually slow a bacterium down

as it approaches the surface. An additional fluid drainage force results as the bacterium encounters resistance near the wall due to pressure in the draining fluid film between the wall and the approaching bacterial surface. Kent (120) described downsweeps of fluid from the turbulent core as the most important mechanism of transport to surfaces. These turbulent downsweeps provide a significant fluid mechanical force that directs bacteria toward the surface. Lift, drag, and drainage forces help to elucidate some of the transport mechanisms involved in the process of bacterial deposition.

II. Reversible Adhesion

Reversible adhesion is an instantaneous attraction between bacteria and surface by long range forces (142). Reversible sorption is the initial phase in the process of bacterial attachment when the bacterium is weakly held at the surface. Bacteria continue to exhibit Brownian motion and can be easily removed from the surface by washing with 2.5 % NaCl or by the violent rotational movements of some motile bacteria (74, 142).

There are basically three types of long-range interactions between surfaces: van der Waals, electrostatic, and steric forces. The DLVO theory, named for the researchers who described it, explains the observed behavior of microorganisms when they approach surface. The DLVO theory, developed independently by Derjaguin and Landau, and Verwey and Overbeek, relates the stability of colloidal

dispersions to the total potential energy of interaction between two particles. This theory is more completely detailed in section C below.

A. Electrostatic Forces

When immersed in an aqueous solution, solids acquire a surface charge. This occurs either by ion adsorption or by ionization of surface groups. Under normal conditions, most surfaces assume a negative charge. However, this will depend on the composition of the aqueous phase (97). Most bacterial surfaces are composed of acidic groups (carboxyl or phosphate) and basic groups (amino) and at low pH values the bacteria could carry a net positive charge. A charged solid surface will attract oppositely charged ions from the aqueous phase and repel like charged ions away from the surface. This, together with the opposing effects of thermal motion, distributes the ions in a Poisson-Boltzman distribution throughout the aqueous phase and leads to an imbalance of positive and negative ions in the region of the charged surface (97). In this region, there is an unequal balance of positive and negative ions, this is generally referred to as the diffuse electrical double layer.

B. Steric Forces

Biological systems often contain a variety of macromolecules (glycoproteins, proteoglycans) and adsorption of these macromolecules occurs almost immediately.

These macromolecules have a significant effect on particle interactions and hence, can play a major role in the attachment of microorganisms to surfaces (97). The adsorbed layer of macromolecules is often referred to as the conditioning film.

C. DLVO Theory

In the natural environment, most solid surfaces possess a net negative surface charge. Oppositely charged ions are loosely attracted to the surface to form a diffuse double layer of ions. When two negatively charged particles are in close proximity, they may be repelled from or attracted to each other (142). Marshall (142) stated that this effect depends on the thickness of the diffuse double layer which, in turn, is dependent on valency and concentration of the electrolyte. Particle interaction can be predicted by the colloid stability, or DLVO theory, developed independently by Derjaquin and Landau and Verwey and Overbeek. The DLVO theory accounts for the stability of hydrophobic colloidal particles of like charges in a aqueous medium. According to this theory, adhesion depends on a balance of short-range forces and longer electrostatic repulsive forces (74). Fletcher (74) stated that the DLVO theory can predict a strong attraction between surfaces at very close range. According to Jones and Isaacson (117), the DLVO theory states that as two particles with like charges approach each other they encounter attractive and repulsive forces which are additive in effect and vary independently with the distance of separation. They explain that at long distances

of separation (> 10 nm) the forces of attraction are the greatest and the particles are held in a state of mutual attraction. The authors described this region as the secondary minimum of potential energy. Here the forces of attraction are weak and may be reversed by mild fluid forces. At shorter distances (between 1 and 10 nm), the adsorbed counter ion clouds cause the particles to repel. Jones and Isaacson (117) revealed that if these forces of potential energy maximum can be overcome and even shorter distances of separation reached (< 1 nm), then the forces of attraction are great and the particles are held in a state of irreversible attachment by the forces in the primary minimum of potential energy.

The potential energy of interaction at increasing distances between bacterium and a solid surface can be estimated from the magnitude of the London-van der Waals attractive energies and the electrical repulsion energies in the overlapping double layers surrounding the bacterial and substratum (142). The derivation of the DLVO theory employed is applicable only to the analysis of reversible, weak long-range interactions. A secondary attraction minimum is apparent at smaller double layer thickness values. Bacteria should be attracted reversibly to the secondary minimum at high electrolyte concentrations, but should be repulsed from the surface at lower electrolyte concentrations (142). The magnitude of the repulsion barrier depends on the ionic composition and charge of the medium and the radii of curvature of the two surfaces (74).

When the bacterium, which is negatively charged, comes into contact with

the negatively charged substratum, the bacterium is unable to overcome the repulsion barrier that occurs at very small interparticle distances. The bacterial cell is, therefore, held at some small, but finite, distance from the surface. The bacterium held by forces at the secondary minimum is now able to irreversibly attach to the surface by means of extracellular appendages if shear forces are low.

III. Irreversible Adhesion

The long range forces determine if a microorganism will be able to contact the surface. However, once in contact with the solid surface, the microorganism interacts by means of short range forces. Bacteria in close proximity to a surface may bind firmly through a number of extracellular polymeric components external to the cell envelope which typically include pili, fimbriae, and flagella. The type of extracellular polymer present is dependent upon the bacterial species and its physiological state (97).

As early as 1943, ZoBell (235) suggested that extracellular adhesive materials were responsible for firm attachment of bacteria to surfaces. Hirsch and Pankratz immersed electron microscope grids in aquatic environments and discovered that bacteria were capable of producing amorphous, granular, and fibrous "holdfast" structures (142). Marshall, et al. (144) described irreversible attachment as a time-dependent firm adhesion whereby the bacteria no longer exhibit Brownian motion and cannot be removed by washing. They suggested that

extracellular polymer bridging was responsible for the firm anchoring of bacteria to a surface in response to nutrient accumulation.

These polymeric components are produced at the primary minimum and facilitate firm attachment as a result of the high attractive energies. At short distances, short-range forces become especially important. The short range forces (distances less than 0.4 nm) can be loosely classified into three types: chemical bonds which include electrostatic, covalent, and hydrogen bonds; dipole interactions which include dipole-dipole interactions, dipole-induced dipole, and ion-dipole interactions; and solvation forces which includes hydration forces and hydrophobic bonding (97).

Once attached, the bacterium may produce additional pellicle proteins which create new receptor sites for other cells (positive cooperativity) (142). This is an important aspect to consider in natural habitats because inert surfaces may become conditioned by adsorbed molecules from the aqueous phase. The conditioned surface then becomes the site of interaction instead of the pristine substratum.

Fletcher (74) stated that there are unsatisfied bonds at the solid surface. This potential bonding energy is represented by surface free energy. Dispersion, dipole, electrostatic, and metallic forces contribute to the surface free energy. Hydrophobic cells appear to adhere more firmly than do hydrophilic bacteria. Fletcher and Loeb (76, 77) found that a marine pseudomonad attached at higher

levels to hydrophobic surfaces. They demonstrated a direct relationship between increasing attachment and increasing hydrophobicity (water contact angle, Θw) of the substrata.

IV. Microbiological Aspects of Adhesion

Many microorganisms are motile; they are able to move against fluid flow, a concentration gradient, or along a solid-liquid interface. Since microorganisms and solid surfaces tend to possess a net negative charge in an aquatic environment, colloid chemistry would suggest that the interaction would be net repulsion. A high repulsion energy barrier would prevent direct contact between cell and surface. The short range forces, such as chemical bonding would not be able to overcome the potential energy barrier at around 1 nm from the surface, thus preventing firm attachment. The cell would be expected to get no closer than the "secondary minimum" distance, of around 5-10 nm from the surface (97). Specialized attachment structures and extracellular polymers allow the cell to overcome this barrier and firmly attach to surfaces even when this is not energetically feasible.

A. Specialized Attachment Structures

According to the DLVO theory, the repulsive energy barrier decreases as the radius of the particle decreases. Therefore, a cell could overcome the potential

energy barrier and attach to the surface at the "primary energy minimum" if the cell could reduce its radius of interaction. At this point, attachment would be stronger and more permanent than at the secondary minimum. Many microorganisms produce thin, extracellular filamentous appendages, which would facilitate bacterial attachment because their radius of interaction is far lower than that of the cell itself. In addition, these appendages may have a reduced surface charge compared with the bacterial cell (184).

1. Flagella

Flagella provide motility for some bacteria. These are very fine threads composed of the protein flagellin with a helical structure extending out from the cytoplasm through the cell wall (97). Flagella may have a diameter between 0.01 to 0.02 μm, and a length of up to 10 μm. The action of the flagella is not enough to propel an organism through the potential energy barrier to the primary minimum, but the flagellum itself may form an adhesive bond with the surface.

2. Pili or Fimbriae

These structures are more likely than flagella to play a role in adhesion. These are produced by a variety of microorganisms, especially Gram negative bacteria. They are fine, filamentous proteins, 5 to 25 nm in diameter and up to 1-2 μm in length (228). They are usually straight, and not involved in motility.

Their only known general function is to provide adhesions for the cell. Pearce and Buchanan (176) stated that fimbriation of species is an expression of an ecological advantage for bacteria in natural environments. They suggested that fimbriation was a reversible trait and was most likely controlled by a chromosomal gene. Also, the authors note that fimbriae increase adherence by penetrating the electrostatic barrier by means of its small surface area and radius of curvature. This effect may also be aided by uncharged hydrophobic residues and a decreased charge density (176). According to Ward (228), the amino acid composition of pili indicate the presence of low numbers of basic amino acids, few free carboxyl groups, and a high proportion of residues with hydrocarbon side chains. Ward (228) stated that this composition would favor adhesion due to hydrophobic properties. Piliated cells strongly adhere to other bacterial species, mammalian cells, soil particles, or a variety of inert surfaces.

B. Polymers as Adhesives

Extracellular polymeric materials are secreted by many bacteria. Widely different molecules exist, from the simple homopolysaccharides assembled from identical monomeric units to the more complex heteropolysaccharides and muco-lipoproteinaceous polymers (97). Often, the type of polysaccharide is unique to the species of bacterium. Several types of monomers have been identified in microbial extracellular polymers, including neutral sugars (D-glucose, D-galactose,

D-mannose, L-fucose, L-rhamnose), amino sugars (N-acetyl-D-glucosamine and N-acetyl-D-galactosamine), and uronic acids (D-glucuronic and D-galacturonic acids) (Habron). Habron (97) noted that pentose sugars such as D-ribose and D-xylose are frequent constituents of polymers isolated from yeast and algae, but not usually from bacteria. In addition, many extracellular polysaccharides contain combined phosphate, acetate, formate, or pyruvate, which provides acidic groups for further interactions. Polysaccharides appear to be the major components in the extracellular polymeric structures. Other polymeric materials such as glycoproteins, proteins, and nucleic acids have also been found and may contribute to the attachment process.

Extracellular polymers bridge the repulsion barrier and anchor cells to a surface. ZoBell (235) first suggested that extracellular adhesive materials were responsible for the firm adhesion of bacteria to surfaces. Fletcher and Floodgate (75), Marshall (141), and Marshall and Cruickshank (143) demonstrated this phenomenon in numerous studies. Bacteria were allowed to adhere to surfaces, which were then washed, embedded, and ultrathin sections cut perpendicularly to the original solid-water interface (141).

Costerton, et al. (37) described the production of additional polymeric material following adhesion. Bacteria are capable of multiplication within this polymer matrix to form microcolonies. Some polymers may be produced solely for the purpose of adhesion. Subsequent secondary polymers (75) may function

to reinforce the attached microcolony, produce a conditioned surface which facilitates further colonization by other microorganisms (34), or protect the bacteria from antimicrobial agents. The ability to produce extracellular polymers does not guarantee that adhesion will occur. Brown, et al. (22) and Wardell, et al. (229) demonstrated the presence of attached bacteria in a carbon-limited suspension with no measurable production of extracellular polymers. In a nitrogen-limited growth medium, little adhesion occurred even though extracellular polymer production was common.

C. Composition of Polymers

Analysis of the different polymers produced by a variety of bacteria will help elucidate the mechanisms of polymer binding. Information in this area is limited at this point. The term ***exopolysaccharide*** (EPS) is used to describe a variety of polysaccharides found outside the cell wall. Structural components comprise a large group of sugars with a few acyl groups and, occasionally, inorganic residues such as phosphate (220). Bacterial EPS includes polymers of different physical and chemical structures and can be divided into groups based on their chemistry and conditions of synthesis. EPS polymers may be divided into two main groups, homopolysacccharides and heteropolysaccharides, the former consists mainly of glucans, fructans, or polymers of N-acetylamino sugars (220). Heteropolysaccharides are usually composed of repeating hexoses of the D-series:

the 6-deoxyhexoses, L-rhamnose, and L-fucose; N-acetylamino sugars, and D-hexuronic acids, mostly D-glucuronic acid (220). The glycan portion of the repeating unit structure is thought to be constant. Structural diversity arises because noncarbohydrate components are often present in nonstoichiometric amounts and can vary considerably depending on growth conditions or growth phase (34).

Corpe (34) analyzed the polymer of P. atlantica and found mannose, glucose, and galactose, in addition to glucuronic acid and pyruvate. This polymer is unique due to its high content of uronic acid, with a 1:1 ratio of hexose to uronic acid. According to Sutherland (218), most microbial acidic polysaccharides contain approximately 20-25% uronic acid. A polysaccharide isolated from Sphaerotilus natans was composed of approximately equal amounts of fucose, glucose, galactose, and glucuronic acid (142).

Sutherland (218) reported that most of the EPS extracted from 17 marine bacteria contained D-mannose, D-glucose, and D-galactose. He noted an absence of O-acetyl groups from these polymers, even though this component is commonly isolated from nonaquatic bacteria. He suggested that these polymers may contain a high number of 1,3- or 1,4-linkages which would provides rigidity to the molecules.

Marshall (142) described nondialyzable fractions of exudates of Pseudomonas NCMB 2021. He stated that these molecules consist of

approximately 65% carbohydrate and 35% protein. The carbohydrate fraction contains equal amounts of "A-polysaccharide" and "B-polysaccharide." The former is composed of mainly of glucose, galactose, and other unidentified sugars (some with acidic groups, whereas the "B-polysaccharide" is somewhat hydrophobic and possesses N-aceytlglucosamine and several other components (probably a 6-deoxy-sugar and O-acetyl groups).

Danielsson, et al. (48) reported that a marine pseudomonad may be removed from surfaces by treatment with proteolytic enzymes. Fletcher and Marshall (78) used pronase to remove Pseudomonas NCMB 2021 from polystyrene surfaces. The same compound was unable to produce the same effect on glass surfaces, suggesting that different mechanisms are involved in the attachment of bacteria to certain surfaces. Fletcher (74) further demonstrated the polysaccharide composition of exopolymers with sodium periodate and disodium tetraborate. These compounds, which are known to disrupt polysaccharides, were found to inhibit bacterial attachment. Periodate acts to oxidize and cleave adjacent hydroxyl groups. Borate also reacts with adjacent hydroxyl groups to yield negatively charged groups, which may produce cross linkages or mutual electrostatic repulsion to disrupt the polysaccharide.

EPS-producing bacteria have been recovered from a variety of ecological niches and the precise role of the EPS depends on the environment. Most EPS are expendable in the laboratory since their function is protection. Physical properties

of the polysaccharide, such as hydrophobicity, electrostatic charge, and viscosity, may determine the amount of protection given to the cell. A highly hydrated EPS layer protects the bacterial cell from drying and predation by protozoans and macrophages. The polymer matrix surrounding the cell has a significant effect on the diffusion of substances, into and out of the cell. Cells buried beneath layers of EPS may be inaccessible to antimicrobial agents. EPS production, especially in the form of capsules, is common among pathogenic bacteria. The type of EPS, the amount synthesized, and the rate of synthesis can all affect the pathogenicity of an organism, yet the exact mechanisms of action are frequently unknown (232). Capsules seem particularly important in bacteria whose host survival stragedy depends on macrophage evasion. Depending on the organism studied, bacteria synthesize EPS during different growth phases and under a variety of growth conditions. Most repeating unit EPS structures studied have been from Gram neagative bacteria and appear to involve essentially similar synthetic processes.

The term ***glycocalyx*** may be defined as any polysaccharide-containing bacterial surface structure distal to the surface of the outer membrane (lipopolysaccharide containing layer) of Gram negative bacteria, or to the surface of the peptidoglycan (murein) layer of Gram positive cells (37). Costerton, et al. (37) indicated that the glycocalyx represents a hydrophilic extension beyond the charged bacterial surface. These hydrophilic entensions are able to approach a charged surface and come within the range of attractive forces, such as hydrogen

bonding, ion pair formation, dipole-dipole interaction, and van der Waals forces (37). 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 (36).

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 (35, 36).

The glycans produced by the cell are able to adhere to inert surfaces. The glycans are continuously produced, which thickens the glycocalyx and entraps more cells of the same or different species. Although most microorganisms prefer production of one type of glycocalyx, several bacteria synthesize different types of EPS (220). It remains unclear whether more than one polymer is secreted throughout the growth cycle.

The fact that the adhesive properties of many bacteria are lost or modified under *in vitro* conditions suggests either a chromosomal mutation or the loss of a

plasmid. Costerton, et al. (36) cautioned that normal subculturing techniques used in the laboratory select for fast-growing mutants that lack the mucoid extracellular polymer (glycocalyx) found in natural habitats.

Much more information is necessary before there is a clear understanding of the entire attachment process. Our knowledge of the events which occur at the solid-liquid interface is limited due to the lack of information on the chemical composition and physical characteristics of the bridging polymers produced by bacteria (142). Studies on the genetic aspects of adhesion may provide useful information into the control of polymer production by bacteria. Genetic studies will also provide insight into the functional groups of certain polymers that may be involved in the adhesion process. Mechanisms of adhesion are complex interactions of many factors. Rutter and Vincent (189) noted that matters are further complicated by the fact that microorganisms are far from being "ideal" particles. They have neither a simple geometry nor do they have a simple uniform molecular composition. The authors further stated that microorganisms are deformable and internal chemical reactions can lead to molecular changes and at the surface, molecules and ions are constantly being exchanged.

V. Conditioning Films

The attachment of bacteria is greatly influenced by adsorbed organic molecules. This adsorption takes place very quickly and "conditions" the surface

to which bacteria may later attach (177). Bacterial adhesion may also be inhibited by conditioning films. Fletcher (73) demonstrated the inhibited attachment of a marine pseudomonad to polystyrene in the presence of serum albumin, gelatin, and fibrinogen. Meadows (156) found similar results with albumin, but showed increased attachment to glass surfaces in the presence of casein and gelatin.

Kent (120) showed that within minutes of exposure, a surface will have adsorbed a monolayer of organic molecules. He states that this layer is usually $>$ 0.1 μm in thickness, but this is enough to condition the surface by significantly altering many of its properties including wettability and surface charge. The nature of this conditioning film may affect the type and extent of microbial interaction with the surface. The surface initially encountered by a cell in the adhesion process will not be pristine but altered in some way.

VI. Factors Influencing Adhesion

Roper and Marshall (188) demonstrated the desorption of attached bacteria from saline sediments exposed to various electrolyte concentrations. By lowering the electrolyte concentration, they showed that some of the natural population of the sediment could be removed. At high saline values, few bacteria were removed, but when the sediment was exposed to successive distilled water rinses, large numbers of bacteria were recovered. This shows the reversible attraction of many bacteria in saline sediments. These bacteria are repelled from

these surfaces at low electrolyte concentrations. In some natural ecosystems, some bacteria may be unable to form irreversible adhesions, but they are still capable of reversible attachment.

Sutherland (219) also discussed the effect of electrolyte concentration on attachment. He cited a study using Al^{3+} and La^{3+} in concentrations of 17 to 200 μm. The author revealed that increasing cation concentrations resulted in a progressive decrease in adhesion. Sutherland (219) described the importance of Ca^{++} and Mg^{++} in adhesion. When grown in a cation rich growth medium, EPS production was abundant, however, when transferred to a cation-deficient medium, secondary polysaccharides were greatly disrupted. The author suggested that although initial adhesion of bacteria to glass was unaffected by the lack of divalent cations, their absence prevented the production of irreversible adhesions responsible for firm attachment.

McEldowney and Fletcher (151) suggested that changes in nutrient conditions in natural aquatic ecosystems will affect the attachment of bacterial species differently, thereby influencing the composition of the developing biofilm. They discovered different patterns of bacterial adhesion by Pseudomonas fluorescens, Enterobacter cloacae, Chromobacterium sp. and Flexibacter sp. in response to changes in growth rate, nutrient conditions, substratum, and organic molecules present. They noted that these results underline the major difficulties in the extrapolation of laboratory data to environmental conditions.

ATTACHMENT TO FOOD CONTACT SURFACES

The surfaces of processing, transport, and storage equipment which contact foods are major sources of contamination (56). Microorganisms attached to food-contact surfaces after cleaning and sanitizing, exist in a complex environment where surfaces, soil residues, detergent residues, moisture, temperature, number of microorganisms, and various other factors influence each other (30). Dunsmore, et al. (57) stated that an organism may attach to surfaces of equipment by: (a) direct attachment, (b) attachment to soil which is attached to a surface, or (c) incorporation in a soil mass which is attached to a surface. Cleaning systems are implemented in food processing plants to produce a safe and wholesome product. Product quality and health hazard concerns exist because many pathogenic and spoilage bacteria are capable of attachment to materials commonly used in food processing equipment. Attached microcolonies may increase in number and contaminate future products. Dunsmore, et al. (57) suggested a possible sequence of contamination that includes: deposition and attachment of cells to equipment surfaces, reduction of bacteria numbers by the cleaning system, proliferation of the survivors during the nonuse period, and contamination of the product as it passes over the contaminated surfaces.

I. Meat Surfaces

Most researchers agree that a linear relationship exists between the number of bacteria in the attachment medium and the extent of bacterial attachment. Notermans and Kampelmacher (171) exposed broiler skins to various concentrations of Pseudomonas putrefaciens in a buffered solution. The bacteria removed after rinsing were considered reversibly attached. They reported that the number of attached bacteria was dependent on type and concentration of bacteria, and type of meat surface. In another study, these authors found that the number of attached bacteria per cm^2 was proportional to the number of bacteria per ml media (170).

Similar results were reported by Butler, et al. (26) who found a direct relationship between attached bacteria and concentration of cells in the attachment medium. In addition, they revealed that the extent of attachment was species dependent. They found that E. coli and Pseudomonas fluorescens showed increases in attachment during 1-30 minutes of exposure, while Lactobacillus sp. and Staphylococcus sp. showed little increase after the first minute of exposure. Similar conclusions were reached by McMeekin and Thomas (155) who reported that microbial contamination of chicken skin depended mainly on bacterial population of the suspension. They found no linear relationship between retention and contact time using E. coli, Flavobacterium, and Micrococcus sp. but, a linear relationship was established using Pseudomonas sp.

Lilliard (133) found that as immersion time increased, the percentage of bacteria in a surface film decreased, while the percentage of bacteria attached to the skin increased. After 0.25 minutes of exposure, 94% of the bacteria were found in the surface film and 6% were on the skin. After 60 minutes of immersion, the number of bacteria changed to 39% in the surface film and 61% attached to the skin. The author suggested that carcass contamination could be reduced if the surface tension of the film could be altered.

Firstenberg-Eden, et al. (66) found that the type of muscle affected attachment. The authors demonstrated that certain bacterial strains attach well to smooth chicken breast muscle, while on other surfaces such cut chicken and beef muscle attachment stopped after 20-30 minutes. In contrast, Butler, et al. (26) discovered that only minor differences in attachment occurred between pork skin, and beef and lamb muscle.

Thomas and McMeekin (222) demonstrated that the attachment of Salmonella spp. to chicken muscle is affected by the ionic environment. They reported that the presence of sodium chloride in the suspension medium prevented firm attachment. In addition, they discovered that altering the ionic environment facilitated the removal of attached bacteria. Saline rinses removed large numbers of cells, while extended rinses in water removed relatively few organisms. This demonstrates the physicochemical relationship involved in the attachment process.

Various factors such as pH, temperature, and motility are also thought to

influence bacterial adherence to surfaces. Notermans and Kampelmacher (171), Butler, et al. (26), and Farber and Idziak (64) suggested that motility is important in bacterial attachment to meat surfaces. Butler, et al. (26) stated that Gram negative motile bacteria showed greater attachment than Gram positive non-motile species. Farber and Idziak (64) demonstrated similar results with low attachment for two non-motile bacteria, Acintobacter and Moraxella osloensis, and the greatest attachment values occurring with Pseudomonas putida and Pseudomonas fluorescens. They also reported that although the non-motile bacteria had the lowest attachment value, these organisms had the highest strength of attachment (S-value). In contrast, other authors concluded that motility of organisms is independent of their ability to attach to chicken surfaces. According to Lilliard (132), all motile and nonmotile test species adhered to poultry skin, and there was a linear increase in rate of attachment with exposure time (0.25 to 60 minutes). Lilliard tested Salmonella typhimurium, Salmonella gallinarium, Proteus vulgaris, Pseudomonas fluorescens, Clostridium perfringens, Staphylococcus aureus, and a nonflagellated species of Micrococcus and concluded that the nonflagellated species attached as readily as flagellated species under the same conditions. Thomas and McMeekin (222) reported that the motility status of Salmonella typhimurium and S. singapore did not significantly affect attachment.

Notermans and Kampelmacher (170) determined that pH and temperature affected the rate and extent of attachment to meat surfaces of certain strains of

bacteria. Butler, et al. (26) reported that pH and temperature of the attachment medium had little effect on the extent of bacterial adhesion. The authors used pH levels ranging from 4.0 to 8.8 and found that no particular pH value seemed to favor the attachment of the five organisms tested.

Firstenberg-Eden (67) stated that attachment allows low numbers of bacteria in poultry rinse water to become concentrated on the meat surface. With time, these bacteria become firmly attached to the meat surface and are difficult to remove. She also revealed that these firmly attached bacteria may be less dangerous to the consumer. Loosely attached bacteria may be spread throughout the kitchen causing increased risk of contamination.

II. Dairy Processing Equipment

Dairy processing equipment surfaces have been shown to be harborages for pathogenic and spoilage bacteria. The presence of attached bacteria is cause for concern since they may contaminate future product that passes over the surface. Most pasteurized dairy products receive no further processing prior to consumption, therefore, pathogenic bacteria capable of rapid growth at refrigeration temperatures are a serious threat.

Studies by Czechowski (44, 45, 46) have shown that bacteria readily attach to food processing equipment. He observed 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 (44). By doing in vivo testing, he demonstrated that gasket materials left in place for extended periods of time become heavily contaminated and may serve as a potential harborage for bacteria (45). Czechowski (46) showed that a high number of bacteria in the environment enhance the potential for attachment and subsequent formation of hard to remove biofilms. He stated that low cell concentrations are also cause for concern because attachment is still evident and cleaning and sanitizing should be designed to reduce even small numbers of bacteria on surfaces. Maxcy (149) demonstrated the complexity of biofilm formation by the use of a model system simulating commercial cleaned-in-place (CIP) conditions. He stated that contaminates in the milk were not the result of a simple contamination, but a complex harborage system. He indicated little difference between acid and alkaline cleaning systems. Maxcy and Shahani (150) noted that contaminating organisms not only result from a simple deposition/growth/recovery cycle, but also occur as a consequence of bacterial harborages such as gasket material.

Herald and Zottola (106) demonstrated the attachment of Yersinia enterocolitica to stainless steel using scanning electron microscopy. Cells adhered at 10, 21, and 35°C but the greatest number of adhered cells occurred at pH 8 and 21°C. They suggested that the adherence may be related to flagella and

surrounding exopolymers. Stone and Zottola (216) showed the presence of Pseudomonas fragi attachment fibrils within 24 h at 21°C and 96 h at 4°C on a stainless steel surface. They noted that an inadequate cleaning procedure would allow residual microorganisms to remain in a processing system. They suggested that significant contamination of the surface could occur if the intercycle (nonuse) period for equipment exceeds 8 h. Residual microorganisms remain viable during the nonuse period and may contaminate product when production resumes.

According to Chatuverdi and Maxcy (30), the soil of food-contact surfaces is composed of food product residues, detergent residues and various ions from the rinse water. This soil, which may support microbial growth, can accumulate through successive contamination and cleaning cycles. These authors noted that sanitizers differed in their surface bactericidal activity and these differences were further related to the type of surface to which they were applied. Speers, et al. (207) failed to demonstrate the accumulation of cells after repeated inoculation and washing steps. They suggested that the secondary layer of cells, those not directly attached to a surface, were more easily removed. Through scanning electron microscopy, these authors identified several factors which facilitate attachment, such as crevices in stainless steel, milk deposits, and production of extracellular material. Lewis and Gilmour (131) sampled sections of rubber and stainless steel which were soiled with fresh raw milk over a 5 d. period. They determined that the residual adherent population was not reflective of the

microflora of the milk and Acinetobacter was found to predominate. In general, the adherent flora contained a mixture of organisms in which Gram positive bacteria predominated even though the flora of the raw milk was primarily Gram negative. They suggested that the Acinetobacter might possess a selective attachment advantage over other microorganisms.

Stanley (213) observed that when stainless steel was exposed to Pseudomonas aeruginosa, cells began to attach in < 1 minute and attachment increased with time. She revealed that attachment of motile cells was reduced 90% by agitation, while the nonmotile cells were unaffected. The author emphasized the importance of flagella to attachment. Removal of flagella resulted in a 90% decrease in adherence. Ionic concentration of the growth medium was shown to affect adherence. Both motile and nonmotile bacteria attached poorly in distilled water and adherence of these organisms escalated as $CaCl_2$ or NaCl concentrations increased to 10mM. At 100 mM, these effects were reversed and attachment decreased. Adherence was maximal at pH 7 to 8 and nonviable cells were shown to adhere at low pH. Attachment of nonviable cells to food contact surfaces are not an immediate threat to product quality or safety, but they may provide a primary layer of attachment to which other cells may adhere.

III. Miscellaneous Food-Contact Surfaces

Dunsmore and Bates (56) reported that the rate of attachment is greatly

influenced by species, growth phase, number of organisms in the bulk phase, temperature, pH, and electrolyte concentration. The authors found considerable differences in the adherence of microorganisms to glass. Pseudomonas aeruginosa attached at a high rate up to 1.5 h, slow attachment continued from 1.5 to 12 h, reaching a maximum density of 2.2 x 10^4 cfu/cm^2, the highest of all 5 organisms tested. Enterobacter aerogenes did not adhere in the first 60 min, but showed slow adherence up to 6 h and an increased rate in 6-12 h, reaching 1750 cfu/cm^2.

Mafu, et al. (137) indicated that Listeria monocytogenes could attach to stainless steel, glass, polypropylene, and rubber surfaces after contact times as short as 20 min. Extracellular materials were evident on these surfaces especially polypropylene at 4°C and glass at 20°C using scanning electron microscopy. Johnson and Lewis (113) investigated the soil retention and harborage of Staphylococcus aureus on dinnerware. The authors suggested that these utensils may harbor accumulations of food and other residues which support bacterial growth. They found, however, that proper application of detergent and dishwasher procedures were sufficient to eliminate this organism from surfaces.

Spurlock and Zottola (211) indicated that cast iron drains in processing plants can support the growth and attachment of L. monocytogenes. They found that L. monocytogenes could survive in cast iron drains regardless of pH or growth media for 28 d. Biofilm formation within floor drains may serve as a potential

reservoir for L. monocytogenes. The authors suggested that drains should not be cleaned with high pressure hoses, due to the potential aerosolization of L. monocytogenes in the food processing plant environment.

RESISTANCE TO ANTIMICROBIAL AGENTS

Most researchers agree that adherence to solid surfaces provides increased protection for the cells within the biofilm. While attached, the exopolysaccharide matrix provides a homeostatic state and minimizes environmental fluctuations. The glycocalyx accomplishes this by concentrating nutrients, and protecting embedded cells from surfactants, antimicrobial agents, and phagocytic cells (179).

The widespread use of cleaned-in-place (CIP) systems increases the risk of complacency toward cleaning and sanitizing because visualization is impossible. Maxcy (148) stated that CIP systems present a problem in that the equipment is not accessible for inspection. He emphasized that residual soils, which may support microbial growth, are likely to be concentrated in trouble spots rather than uniformly distributed throughout the system. Examinations for microbial growth have suggested that CIP joints provide one such harborage. In addition, pump seals and demountable couplings may also be areas of concern. The author suggested that when properly designed, equipment can be cleaned to a point where soil residues are undetectable. A proper cleaning regime prevents accumulation of soil films which support microbial growth.

Gristina, et al. (96) suggested that the anionic, extracapsular, polysaccharide slime produced by bacteria provide an increased resistance to antimicrobials and sequesters critical ions. They found that Staphylococcus

epidermidis cells attached to stainless steel exhibited resistance to normal in-use concentrations of tobramycin. After 8 h exposure to S. epidermidis, 1.2×10^4 cfu/cm^2 were recovered. Exposure of the biofilm to 10 μg/ml tobramycin resulted in only a 2 log reduction with at least 2×10^2 cfu/cm^2 remaining viable after 8 h. The authors suggested that the exopolysaccharides act as an ion-exchange resin whose binding sites must be satisfied before a charged molecule such as an antibiotic can penetrate the glycocalyx.

LeChevallier, et al. (127) showed that the attachment of bacteria provides an increase in disinfection resistance. They found that Klebsiella pneumoniae attached to glass slides exhibited as much as a 150 fold increase in resistance. They noted that other factors which increase resistance include the age of the biofilm, bacterial encapsulation, and previous growth conditions (temperature, pH, growth medium). These factors increased resistance to chlorine from 2-10 fold. The authors found that biofilms grown for 7 d were more chlorine resistant than 2 d biofilms under the same conditions. Like LeChevallier, et al., Brown and co-workers (21) warned against the assumption that resistance patterns are due solely to biofilm development. They observed that nutrient deprivation and growth rate will contribute to antibiotic resistance of biofilm cells.

Stone and Zottola (216) demonstrated that under normal CIP conditions sessile cells did not exhibit attachment fibrils. If suboptimal CIP conditions were used, these cells displayed attachment fibrils. The authors observed that organisms

remaining after normal CIP were not viable, while those present after suboptimal conditions remain viable. Attachment was observed within the first 1/2 h of circulation of inoculated milk in the plug area, which represents a dead end with the slowest rate of flow. Under normal cleaning conditions, most attached cells were removed in the post-soiling rinse. They found no difference in the ability of two CIP cleaning procedures, acid and alkaline detergent, to remove attached bacteria when normal concentrations of detergent and sanitizer were used at recommended temperatures.

McEldowney and Fletcher (152) studied the survival of bacteria attached to dry glass surfaces. They found the survival of Gram positive and Gram negative bacteria to be similar. Survival at 0%, 34%, and 75% relative humidity was shortest at 25°C and longest at 4°C. The results of this study indicated that survival of bacteria on dry processing and container surfaces may provide a contamination hazard for extended periods. Most bacteria tested were able to survive but could not perform normal cell functions. However, Pseudomonas sp underwent cell division even at 0% relative humidity. The authors suggested that 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.

Mafu, et al. (138) demonstrated that the attachment of L. monocytogenes

to stainless steel, glass, polypropylene, and rubber. They indicated that sanitizers were more effective against L. monocytogenes attached to nonporous surfaces. After 10 min of contact time, the limit concentration of sanitizer was 5-10 times greater for rubber than stainless steel and glass. Higher concentrations of sanitizer were required at 4°C than 20°C. In further studies with L. monocytogenes, Frank and Koffi (82) demonstrated an increased resistance of cells attached to glass slides. After an initial 2-3 log decrease, the remaining sessile bacteria survived 20 min of exposure to benzalkonium chloride (100, 400 and 800 ppm) and acid anionic sanitizer (200 and 400 ppm). After 30 s of exposure to the lowest concentration of sanitizers, planktonic cells were reduced to undetectable levels. They also reported that adherent cells exhibited less than a 5 log decrease when heated for 5 min at 70°C. Similar to the results of LeChevallier, et al. (127), Lee and Frank (129) showed that 8 day adherent L. monocytogenes were 100 times more resistant than 4 day adherent cells when exposed to 200 ppm hypochlorite for 30s. When stainless steel slides with adherent microcolonies were heated to 72°C, both 4 d and 8 d biofilms were inactivated after 1 minute. Detectable cells remained after 3 min of exposure to 65°C when 8 d adherent cells were used. The same results were not found for 4 d cells.

CONSEQUENCES OF ADHESION

I. Benefits for the Bacterium

Attachment to a solid surface provides the bacterium with an environment rich in adsorbed macromolecular nutrients in the surrounding nutrient poor aqueous phase. Also, attachment could provide more favorable physicochemical environmental conditions, such as an altered pH or oxygen tension. Cells attached to solid surfaces gain a survival advantage, in that the development of a thick slime layer provides a microcolony environment which is protected from fluctuating, and often stressful, conditions in the surrounding medium (74). Nutrient diffusion limits bacterial growth and metabolism within the matrix, but some bacteria survive until they are sloughed off into the medium. The biofilm acts as a bacterial reservoir capable of repopulating the aquatic environment when conditions favor growth (74).

Bacteria adhere to inert surfaces in order to maintain a homeostatic environment. Costerton, et al. (35) found that a submerged surface may typically have 10^6 bacteria attached to it, while the aqueous phase contains 10^3 cells/cm^3. Cells thrive on the nutrients extracted from the bulk liquid. A stationary location provides a continuous supply of organic nutrients, vigorous aeration, and waste removal. The extracellular polysaccharides position the cells within the biofilm, and function to conserve and concentrate enzymes released by the bacteria, thus

producing an immobilized enzyme system. Also, the glycocalyx functions as a nutrient reservoir. The polysaccharide fibers are mostly negatively charged and act as an ion-exchange resin. Suspended ions and molecules are trapped and made available to the cells within the biomatrix.

Biofilms reduce the effectiveness of many antimicrobial agents such as macrophages, antibodies, sanitizers, and antibiotics. The glycocalyx provides a penetration barrier for the attached bacteria. Biofilms provide nutrients for growth by capturing inorganic and organic molecules from the aqueous phase. Within the biofilm, the glycocalyx holds cells in close proximity, which ensures an exchange of metabolites, removal of toxins, and combination of enzymatic activities from several species (38).

II. Deleterious Effects

Biofilms may influence fluid flow behavior by restricting the effective flow diameter, causing reductions in flow. For example, Kent (120) stated that a 1.5 mm biofilm on a 12.7 mm diameter pipe will reduce the flow area by 42%. In addition, extensive biofilm formation may result in severe blockage or uneven flow distribution. This uneven flow can cause pressure drops within flowing systems, which result in increased pumping energy requirements or decreases in fluid-carrying capacity.

Kent (120) described a report from a World War II Admiralty which

estimated that 6 months' cruising in temperate waters at 20 knots produced enough biofouling on the hull of a battleship to increase fuel consumption at that speed by 40%. Bacterial metabolism within a biofilm establishes a corrosion potential, which can penetrate a 5/8"-thick steel plate in less than 6 months (38). Therefore, biofilms can increase pumping costs, reduce heat exchanger efficiency, and add to equipment replacement costs as a result of corrosion. Biofouling eventually increases vessel drag, reducing speed and increasing fuel consumption (38).

Economic and energy losses resulting from biofilm formation may become extensive if ignored. According to Characklis (29), biofilm formation may result in energy losses due to increased fluid frictional resistance as well as increased heat transfer resistance. He described a documented case of bifouling at a nuclear plant in which the heat transfer rate was decreased by 30% over a 30 day period. Adhesion of microorganisms increases capital costs for excess equipment capacity which compensates for fouling. Economic losses occur when equipment must be prematurely replaced. Characklis (38) described a nuclear power plant condenser which had to be replaced after 6 years of use when it was designed to last 20. Unexpected downtime and subsequent loss of production due to biofilm formation resulted in dramatic economic losses. The author presented an estimate of economic losses for Britain at $600-1,000 million per year (approximately 0.5% of the British 1976 GNP).

Biofilms have the potential to cause health hazards and quality control problems. 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. Equipment of drinking water distribution systems may also become fouled and contaminate tap water, which may affect public health. Characklis (29) noted that some deaths due to Legionnaire's disease have been attributed to the fouling of cooling water towers.

Within the biomatrix, bacteria are continually growing, multipling, and lysing. The surfaces to which these bacteria are attached may be damaged or fouled by these metabolic processes. In marine environments, biofilm formation is merely a precursor to colonization by more complex organisms which feed on the attached bacteria. Biofilms may serve as a reservoir of pathogenic bacteria and cause material deterioration, such as corrosion of metals and dental decay. Costerton and Lappin-Scott (38) reported that regular chlorination of drinking water reduces bacterial numbers to permitted levels, but does not kill reservoirs of attached bacteria on the pipeline surface, which may periodically detach, raising bacterial numbers to unacceptable levels.

MATERIALS AND METHODS

I. Test Organisms

Pseudomonas fluorescens ATCC 13525 was grown on Nutrient Agar (NA) (BBL Microbiology Systems, Cockeysville, MD) slants at 21°C. Yersinia enterocolitica ATCC 9610, Bacillus cereus ATCC 11778, and Listeria monocytogenes Strain Scott A serotype 4b were grown on Trypticase-soy Agar + 0.6% yeast extract (TSYA) (BBL Microbiology Systems, Cockeysville, MD) slants at 32°C. Transfers were made on a monthly basis. The inoculum was subcultured into nutrient broth (NB) for Pseudomonas, Yersinia, Bacillus, and T-soy + 0.6% yeast extract broth (TSYB) for Listeria on three consecutive days, prior to each experiment, to ensure an actively growing culture.

II. Test Materials

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 rinsed with water, wrapped in aluminum foil and sterilized for 15 minutes at 121°C.

III. Sanitizer Efficacy Test

The actively growing culture of each organism was inoculated onto slants of the appropriate medium described above and incubated overnight. The test organism was removed from the slants with 5 ml of phosphate buffer and sterile 4 mm glass beads. The suspension was poured into a 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 French bottle flats that were prepared by the following method. Twenty ml of sterile medium was placed into a sterile 250 ml French square bottle. The media and temperatures used for growth were nutrient agar (NA) for P. fluorescens at 21°C; NA for Y. enterocolitica, and B. cereus at 32°C; and TYSA for L. monocytogenes at 37°C. The bottle was placed horizontally for solidification of the medium. After 15 to 20 minutes, the excess liquid was poured off and the flats were incubated at the appropriate growth temperature for 48 hours.

Two ml of phosphate buffer and glass beads were added to each flat to "strip" the bacterial growth from the medium. This suspension was poured into a buchner funnel with Whatman No. 2 paper. The colonies were broken up by vacuum filtration. A Spectronic 21 (Milton Roy Co., WA) was used at 540 nm to standardize each culture to 10^{10} cfu/ml. The test suspensions were diluted to obtain readings from the spectrophotometer in the 0.4 to 0.6 range. Plate counts, using the media and incubation temperatures described above, were

performed on the suspension to confirm the presence of 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, 1 ml was removed and placed in 9 ml neutralizer blanks described below for 5 $\pm$ 1 min. Following the inactivation period, the number of surviving organisms were determined using the pour plate method (108). The media and incubation temperatures used for enumeration were nutrient agar (NA) for P. fluorescens at 21°C; NA for Y. enterocolitica, and B. cereus at 32°C; and TYSA for L. monocytogenes at 37°C.

Six sanitizers were used in the following part per million concentration (ppm) of their active ingredient: iodophor, 25; chlorine, 200; acid anionic, 200 (Diversey Wyandotte, Wyandotte, MI); peroxyacetic acid, 200; quaternary ammonium compound, 200; and fatty acid, 200 (Klenzade, Division of Ecolab, Inc., St. Paul, MN). These are typical in-use concentrations. The active ingredients of each sanitizer are as follows: (a) chlorine, sodium hypochlorite, 9.2%; (b) iodophor, iodine (from potassium triiodide), 1.75% and phosphoric acid, 15.75%; (c) acid anionic, phosphoric acid 59.8%; (d) peroxyacetic acid, peroxyacetic acid, 4% and hydrogen peroxide, 25%; (e) fatty acid, phosphoric acid, 22.5% and citric acid, 20.0%; (f) QUAT, n-Alkyl [50% C_{14}, 40% C_{12}, 10% C_{16}] dimethyl benzyl ammonium chloride.

Neutralizer blanks were prepared according to AOAC procedures (108) with 10.4 ml of a 0.25 M buffer stock solution (described below), 46.0 ml of AOAC Neutralizer stock (described below), 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. The neutralizer stock solution contained 40 g azolectin, 280 ml polysorbate 80, and 1.25 ml phosphate buffer. This solution was diluted to 1 liter and the pH adjusted to 7.2, then autoclaved at 121°C for 20 minutes. The sanitizer efficacy test was performed in triplicate.

IV. Impedance Microbiology

Impedance measurements were performed using a Bactometer Microbial Monitoring System Model 128 (bioMérieux Vitek, St Louis, MO). As described by Mosteller and Bishop (164), 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 was 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 converted into cfu/ml. 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).

Pseudomonas isolation medium was prepared with peptone (20 g/l), magnesium chloride (1.4 g/l), potassium sulfate (10 g/l), and 20 ml of glycerol. This medium was autoclaved 15 min at 121°C. The following modifications were made: (a) 16 ppm nalidixic acid, (b) 8 ppm ampicilin (c) 8 ppm polymyxin B (Sigma Chemical Co, St. Louis, MO). These modifications were made in order to inhibit the growth of Yersinia and Listeria. Modifications were evaluated with pure cultures to assess the growth of test organisms. Tests were conducted at 21°C for 48 h with 1.5 ml of medium in each well.

Yersinia isolation medium contained yeast extract (2 g/l), peptone (17 g/l), proteose peptone (3 g/l), mannitol (20 g/l), sodium deoxycholate (0.5 g/l), sodium cholate (0.5 g/l), sodium chloride (1 g/l), sodium pyruvate (2 g/l), magnesium sulfate heptahydrate (10 mg/l), neutral red (30 mg/l), and crystal violet (1 mg/l). This medium was autoclaved at 121°C for 15 min. After cooling to 45-50°C, Yersinia CIN Antimicrobial Supplement (Cefsulodin, 7.5 mg/l; Irgasan, 2.0 mg/l; Novobiocin, 1.25 mg/l) (Oxoid, Unipath LTD, Hampshire, England) was added. Modifications to this medium included: (a)

2.5% bile salts, (b) 8 ppm erythromycin (Sigma Chemical Co, St. Louis, MO), (c) pH adjusted to 6.0, (d) pH adjusted to 8.0. These modifications were made to provide a more selective medium for Yersinia enterocolitica. Tests were done with pure cultures to determine the effectiveness of the modifications. A 1.5 ml aliquot of isolation medium was placed into each well and samples were monitored at 32°C for 48 h.

The following modifications were made to Listeria Enrichment Broth (LEB) (Difco Laboratories, Detroit, MI) to produce a more selective medium: (a) 15 g/l lithium chloride (Fisher Scientific, Raleigh, NC), (b) addition of Oxford Antimicrobic Supplement (Difco Laboratories, Detroit, MI). This medium was tested with pure cultures at 25 and 37°C.

V. Efficacy of Sanitizers Towards Attached Mixed Cultures of Bacteria

Ninety-nine ml of two percent milk was steamed for five minutes at 121°C and stored at 2°C until use. Milk blanks were warmed to room temperature and inoculated with 1 ml of a 10^8 cfu/ml suspension of Pseudomonas fluorescens, Yersinia enterocolitica, and Listeria monocytogenes. Gasket pieces and inoculated milk were added to a 250 ml erlenmeyer flask 12 hours at 21°C. To better simulate movement of product through a pipeline, a benchtop shaker (Tekmar, W. Germany) was set at 150 rpm. Samples of the milk suspension were tested for the presence of each organism at the beginning and end of the incubation period.

Selective media and incubation temperatures are described below.

Six sanitizers were used in the same ppm concentrations of active ingredient described in section III. Gasket pieces were removed from the inoculating milk, rinsed with phosphate buffer to remove loosely adhering cells, and exposed to 10 ml of sanitizer solution for 30 seconds with low speed vortexing. After exposure, the gaskets were placed in 9 ml of neutralizer buffer for 5 $\pm$ 1 minutes at 21°C.

A mini sample container was used with a Waring blender Model 700G to dislodge attached cells (Fisher Scientific, Raleigh, NC). Fifteen ml of T-soy broth was placed into the blender along with the gasket piece. After 30 sec of blending, the T-soy broth was removed and bacteria were enumerated. Aliquots were plated using the selective media, described below, and by the repair/detection procedure. Differences in the counts indicated that injured cells were present and unable to grow on the selective media. The repair/detection technique allowed for the enumeration of both injured and noninjured cells. Samples were first plated with 5-6 ml of T-soy agar + 0.6% yeast extract (TYSA). Following a 2 h incubation at 21°C, these plates were overlayed with a selective medium.

Pseudomonas isolation agar (Difco Laboratories, Detroit, MI) + 10 ppm polymyxin B (Sigma Chemical Company, St. Louis, MO) was used to enumerate Pseudomonas fluorescens at 21°C for 48 h (11). Cefsulodin-Irgasan-Novobiocin (CIN) agar (Difco Laboratories, Detroit, MI) was used to enumerate Yersinia enterocolitica at 32°C for 24 h (65). Oxford agar + oxford supplement (Difco

Laboratories, Detroit, MI) was used to count Listeria monocytogenes at 32°C for 24 h (11). Each test organism was plated on the three media to ensure recovery of selected organisms. All tests were conducted in triplicate.

VI. Efficacy Towards Attached Bacteria in a Circulating Pipeline System.

A Masterline Model 2095 circulating water bath was fitted with 7 feet of stainless steel pipe to achieve circular flow of milk to simulate a dairy pipeline system (Forma Scientific, Marietta, OH). The system was rinsed with hot water (60°C) for 10 min prior to the beginning of each experiment. Next, 13.25 liters (3.5 gallons) of freshly pasteurized milk were placed into the pipeline system and inoculated with the test organism. The test organisms for these experiments were Pseudomonas fluorescens, Yersinia enterocolitica, and Bacillus cereus. One ml of each actively growing test culture was placed separately into 300 ml of sterile milk and incubated for 24 h at the previously described growth temperatures. Prior to inoculation into the pipeline system, the inoculated milk was cooled to 10°C for 2 h. The initial number of bacteria in the system was 10^5 cfu/ml. Samples of the milk suspension were tested for the presence of each organism at the beginning and end of the incubation period. Selective media and incubation temperatures for Yersinia and Pseudomonas are described in the preceding section and Bacillus cereus was enumerated using Mannitol-Yolk-Polymyxin (MYP) agar (Difco Laboratories, Detroit, MI) for 24 h at 32°C (103). Milk was circulated in the

pipeline system at 7°C for 48 h at a flow rate of 2.3 liters/min. Gasket sections were suspended in the flowing milk by means of a stainless steel sleeve with the dimensions of 45 mm x 5 mm x 2 mm.

The pipeline system was cleaned using the following system: pre-rinse, 43°C for 1 min; cleaner, 63°C for 10 min; post-rinse 43°C for 1 min; and sanitizer 63°C for 2 min. The sanitizers were used in the ppm concentrations previously described. A chlorinated alkaline CIP cleaner was used in the recommended concentration of 0.5 oz/gal (Diversey Wyandotte, Wyandotte, MI). The CIP cleaner is comprised of sodium hydroxide, sodium hypochlorite, sodium chloride, and sodium polyacrylate.

Gasket sections were removed following each step in the cleaning system and initial numbers of bacteria and survivors were enumerated. After each step, gasket sections were removed from the pipeline system and placed into 9 ml neutralizer buffer. Bacteria were enumerated using the Bactometer Microbial Monitoring System Model 128 (bioMérieux Vitek, St. Louis, MO) as described in section IV. Duplicate replications were conducted.

VII. Efficacy Towards Attached Mixed Cultures of Bacteria in a Pipeline System

The same pipeline system described in the pure culture study was used to

examine mixed cultures of bacteria. In this part of the study, a mixed culture of bacteria was circulated at 7°C for 48 h. Gasket sections were placed in the system as previously described. One ml of <u>Pseudomonas</u> <u>fluorescens</u>, <u>Yersinia</u> <u>enterocolitica</u>, and <u>Bacillus</u> <u>cereus</u> were used to inoculate 3 separate 99 ml milk blanks. After 24 h incubation at the optimum growth temperatures, the inoculated blanks were cooled to 10°C for 2 h and then added to the pipeline system. Following 48 h of circulation at 7°C, gasket sections were removed after each step in the cleaning system mentioned above and placed into 9 ml neutralizer buffer. Attached bacteria were removed using a mini sample container with a Waring blender Model 700G (Fisher Scientific, Raleigh, NC). Fifteen ml of T-soy broth was placed into the blender along with the gasket piece. After 30 sec of blending, the T-soy broth was removed and bacteria were enumerated by the repair/detection method described in section V. The selective medium used for <u>Bacillus</u> <u>cereus</u> was Mannitol-Yolk-Polymyxin (MYP) agar (Difco Laboratories, Detroit, MI) for 24 h at 32°C (103). Tests were performed in duplicate.

Analysis of variance (ANOVA) tests of the data were completed using SAS (SAS Institute, Inc. Cary, NC).

RESULTS AND DISCUSSION

I. Sanitizer Efficacy Test

All six sanitizers used in this study provided a > 5 log reduction in bacterial numbers in a suspension test (Table 1). This demonstrated the effectiveness of sanitizers at in-use concentrations towards pure cultures of bacteria in suspension. Similar results were reported by Lopes (134), who observed a > 5 log reduction of Salmonella typhimurium, and Listeria monocytogenes exposed to dairy and food plant sanitizers at in-use concentrations. Mosteller and Bishop (164) found that Pseudomonas fluorescens, Yersinia enterocolitica, and L. monocytogenes exposed to chlorine, iodophor, acid anionic sanitizer, peracetic acid sanitizer, fatty acid sanitizer, and QUAT at in-use concentrations also exhibited a > 5 log reduction in cell numbers. Bacteria in suspension exist as free floating cells or groups of cells with little or no glycocalyx production which may increase bacterial resistance to antimicrobial agents. Glycocalyx requirements are minimal, since environmental fluctuations are minimal.

II. Efficacy of Sanitizers Towards Attached Mixed Cultures of Bacteria

In a mixed culture consisting of P. fluorescens, Y. enterocolitica, and L. monocytogenes, significant differences existed between the initial attachment of the various organisms, and differences in attachment to the two surfaces were evident

Table 1 - Number of surviving organisms from a suspension test

	CL	IO	AA	PA	FA	Q
Pseudomonas fluorescens (3.7E6)	< 1	< 1	< 1	< 1	< 1	< 1
Yersinia enterocolitica (5.1E6)	< 1	< 1	< 1	< 1	< 1	< 1
Listeria monocytogenes (6.2E6)	< 1	< 1	< 1	< 1	< 1	< 1
Bacillus cereus (1.3E6)	< 1	< 1	< 1	< 1	< 1	< 1

CL = Chlorine (200 ppm) IO = Iodophor (25 ppm) AA = Acid Anionic (200 ppm)
PA = Peracetic Acid (200 ppm) FA = Fatty Acid (200 ppm) Q = QUAT (200 ppm)

(Tables 2-7). The number of each organism present in the milk suspension increased 2 $\log_{10}$ over the 12 h test period. Bacteria were removed from the gasket surfaces using a blender, and a repair/detection technique was used in order to recover cells that may have been injured due to sanitizer or from heat generated during blending. The injured cells were allowed to recover on nonselective solid media before selective media overlays were added.

Y. enterocolitica attached at a significantly different ($P < 0.05$) mean initial levels of 5.78 $\log_{10}$ cells/cm^2 to rubber and 4.79 $\log_{10}$ cells/cm^2 to Teflon®. The type of surface influenced the initial level of bacterial attachment. Data analysis showed that there was a significant interaction between the bacterium and surfaces. This demonstrates the complex situation occurring within the biofilm. Biofilm characteristics are dependent upon species, growth temperature, type of surface, and nutritional environment. Scanning electron microscopy conducted by Gaspar-Rolle (88) showed that the topography of buna-N rubber is irregular, rough, and covered with cracks and crevices, while the Teflon® appeared mostly smooth. This would provide a larger number of harborages sites for the bacterium. Speers, et al. (207) observed that bacteria easily attached to dairy equipment such as rubber, glass, and stainless steel and they suggested that the topography of the surfaces may be instrumental in the process of bacterial attachment.

Surface free energy of the two surfaces may also play an important role in initial attachment differences. Gaspar-Rolle (88) demonstrated that buna-N

Table 2 - Log kill of attached bacteria in mixed culture after 30 s exposure to 200 ppm chlorine (Log cells/cm^2; n = 3)

	INITIAL ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
P. fluorescens	5.79 [a]	4.98 [d]	4.27 [a]	4.98 [a]
Y. enterocolitica	6.10 [b]	4.89 [d]	4.35 [a]	4.52 [a]
L. monocytogenes	4.69 [c]	4.50 [e]	4.30 [a]	4.50 [a]

* Means across each row within a block with different letters are significantly different ($P < 0.05$)

* Means down each column within a block with different letters are significantly different ($P < 0.05$)

Table 3 - Log kill of attached bacteria in mixed culture after 30 s exposure to 25 ppm iodophor (Log cells/cm^2 ; n = 3)

	INITIAL ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
P. fluorescens	5.51[a]	5.00[d]	2.01[a]	4.37[b]
Y. enterocolitica	5.90[b]	4.78[d]	1.73[a]	4.78[b]
L. monocytogenes	5.17[c]	4.36[e]	1.97[a]	4.36[b]

* Means across each row within a block with different letters are significantly different ($P < 0.05$).

* Means down each column within a block with different letters are significantly different ($P < 0.05$).

Table 4 - Log kill of attached bacteria in mixed culture after 30 s exposure to 200 ppm acid anionic sanitizer (Log cells/cm^2 ; n = 3)

	INITIAL ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
P. fluorescens	5.65[a]	5.18[d]	1.22[a]	5.18[b]
Y. enterocolitica	5.85[b]	4.91[d]	1.37[a]	4.52[b]
L. monocytogenes	5.19[c]	4.52[e]	0.8[a]	1.28[a]

* Means across each row within a block with different letters are significantly different ($P < 0.05$)

* Means down each column within a block with different letters are significantly different ($P < 0.05$)

Table 5 - Log kill of attached bacteria in mixed culture after 30 s exposure to 200 ppm peracetic acid sanitizer (Log cells/cm^2 ; n = 3)

	INITIAL ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
P. fluorescens	5.31[a]	4.86[d]	4.66[a]	4.86[a]
Y. enterocolitica	5.63[b]	4.74[d]	5.63[b]	4.74[a]
L. monocytogenes	4.69[c]	4.39[e]	4.69[a]	4.39[a]

* Means across each row within a block with different letters are significantly different ($P < 0.05$)

* Means down each column within a block with different letters are significantly different ($P < 0.05$)

Table 6 - Log kill of attached bacteria in mixed culture after 30 s exposure to 200 ppm fatty acid sanitizer (Log cells/cm ^{2} ; n = 3)

	INITIAL ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
P. fluorescens	5.17 [a]	4.81 [d]	1.51 [a]	4.81 [b]
Y. enterocolitica	5.27 [b]	4.38 [d]	0.83 [a]	4.38 [b]
L. monocytogenes	4.54 [c]	4.55 [e]	0.94 [a]	1.43 [a]

* Means across each row within a block with different letters are significantly different ($P < 0.05$)

* Means down each column within a block with different letters are significantly different ($P < 0.05$)

Table 7 - Log kill of attached bacteria in mixed culture after 30 s exposure to 200 ppm QUAT sanitizer (Log cell/cm^2 ; n = 3)

	INITIAL ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
P. fluorescens	5.40[a]	5.21[d]	2.02[a]	2.88[b]
Y. enterocolitica	5.96[b]	5.04[d]	2.79[b]	3.44[c]
L. monocytogenes	5.13[c]	4.82[e]	3.17[b]	4.48[d]

* Means across each row within a block with different letters are significantly different ($P < 0.05$)

* Means down each column within a block with different letters are significantly different ($P < 0.05$)

rubber and Teflon® have water contact angle measurements of 151.1° and 112°, respectively. This indicates that the rubber surface has less surface free energy and is more hydrophobic. Fletcher and Loeb (76, 77) reported that a marine pseudomonad attached at higher levels to hydrophobic surfaces. They determined that a direct relationship exists between increasing attachment and increasing hydrophobicity (water contact angle, Θ_w) of the substrata.

Increased harborage sites and differences in surface free energy may also help to explain the differences in log kill of the organisms on rubber versus Teflon®. The reduction of bacteria was less on the rubber surface (Tables 2-7). The strength of adherence of the organisms was greater on the rubber surface probably due to the more hydrophobic surface. This may also reflect the sanitizer's inability to penetrate the crevices of the rubber surface. Mafu, et al. (138) demonstrated that an increase in chlorine concentration is necessary to destroy L. monocytogenes attached to rubber surfaces. After 10 minutes of contact time, the minimum inhibitory concentration of the sanitizer is 5-10 times greater for rubber than stainless steel and glass (138).

Overall, the greatest reduction in cell numbers occurred using 200 ppm peracetic acid sanitizer with an average decrease of 4.66 $\log_{10}$ cells/cm^2 for P. fluorescens, 5.63 $\log_{10}$ cells/cm^2 for Y. enterocolitica, and 4.69 for L. monocytogenes attached to rubber (Table 5). The other sanitizers varied in their effectiveness which illustrates the species dependent effects of biofilm formation

(Figures 4-6, Appendix A). Scanning electron microscopy work conducted by Gaspar-Rolle (88) illustrated the biofilm formation of Pseudomonas fragi, Listeria monocytogenes, and Bacillus cereus. Each bacterium was unique in its appearance, amount and rate of glycocalyx production.

Examination of mixed cultures of bacteria provides a better understanding of interactions that can occur in a pipeline system. Bacteria in dairy processing plants do not exist as pure cultures, but as a part of a larger microflora. Growth and attachment of one species may be favorably or adversely influenced by the presence of other adherent cells. One species of bacteria may attach at sites on the surface, providing secondary attachment sites for other species which may not normally adhere to a particular surface. In contrast, a species may inhibit adherence of other types of cells through the production of certain metabolites.

III. Efficacy of Sanitizers Towards Attached Pure Cultures of Bacteria In a Simulated Milk Pipeline System

Impedance microbiology was used to study the biofilm formation in a simulated milk pipeline system using pure cultures of P. fluorescens, Y. enterocolitica, and Bacillus cereus. In order to relate impedance detection times to cfu/cm^2, a standard curve for each bacterium was developed using linear regression (Figures 1-3). The three bacteria adhered at significantly different ($P < 0.05$) levels with P. fluorescens attaching with the highest frequency (Table 8). The

number of Pseudomonas and Yersinia increased from 10^6 to 10^8 over the 48 h test period. B. cereus showed little increase from the initial 10^5 level which could explain the low rates of attachment. This agrees with previous research by Dunsmore and Bates (56) who observed that attachment rates are greatly influenced by species, growth phase, number of organisms in the bulk phase, temperature, pH, and electrolyte concentration.

In terms of the surface material, there was no difference ($P < 0.05$) in the initial attachment of any of the organisms to either rubber or Teflon®. The numbers of attached P. fluorescens were 6.27 $\log_{10}$ cells/cm^2 on rubber and 6.19 $\log_{10}$ cells/cm^2 on Teflon® (Table 8). This suggests that the variations in surface smoothness was not a factor in the pipeline system. Opportunities for contact between bacterium and surface greatly increase when the suspension medium is in flow rather than static.

The cleaning system was equally effective in removing bacteria from both rubber and Teflon® (Tables 9, 11, 13). Each step in this system was shown to be significant ($P < 0.05$) for B. cereus and P. fluorescens. For Y. enterocolitica, the application of cleaning solution was found to be the important step for bacterial reduction (Table 22-33, Appendix B). This demonstrates the importance of each step within the cleaning regime. No one step can be eliminated or overlooked because each step provides significant amounts of bacterial reduction. The rinse and cleaning steps may not destroy the organisms, but merely remove them from

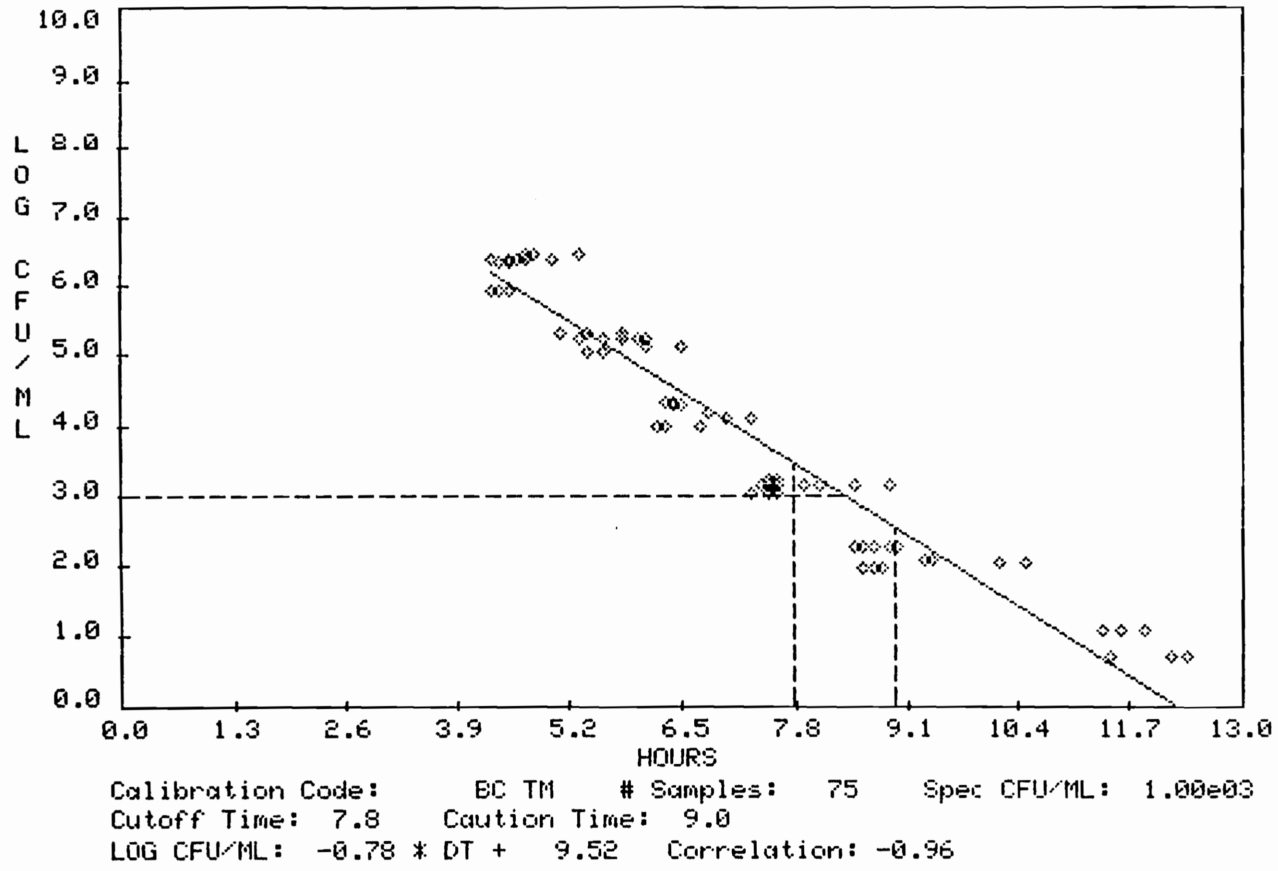


Figure 1 - Standard curve for Bacillus cereus enumeration by impedance microbiology

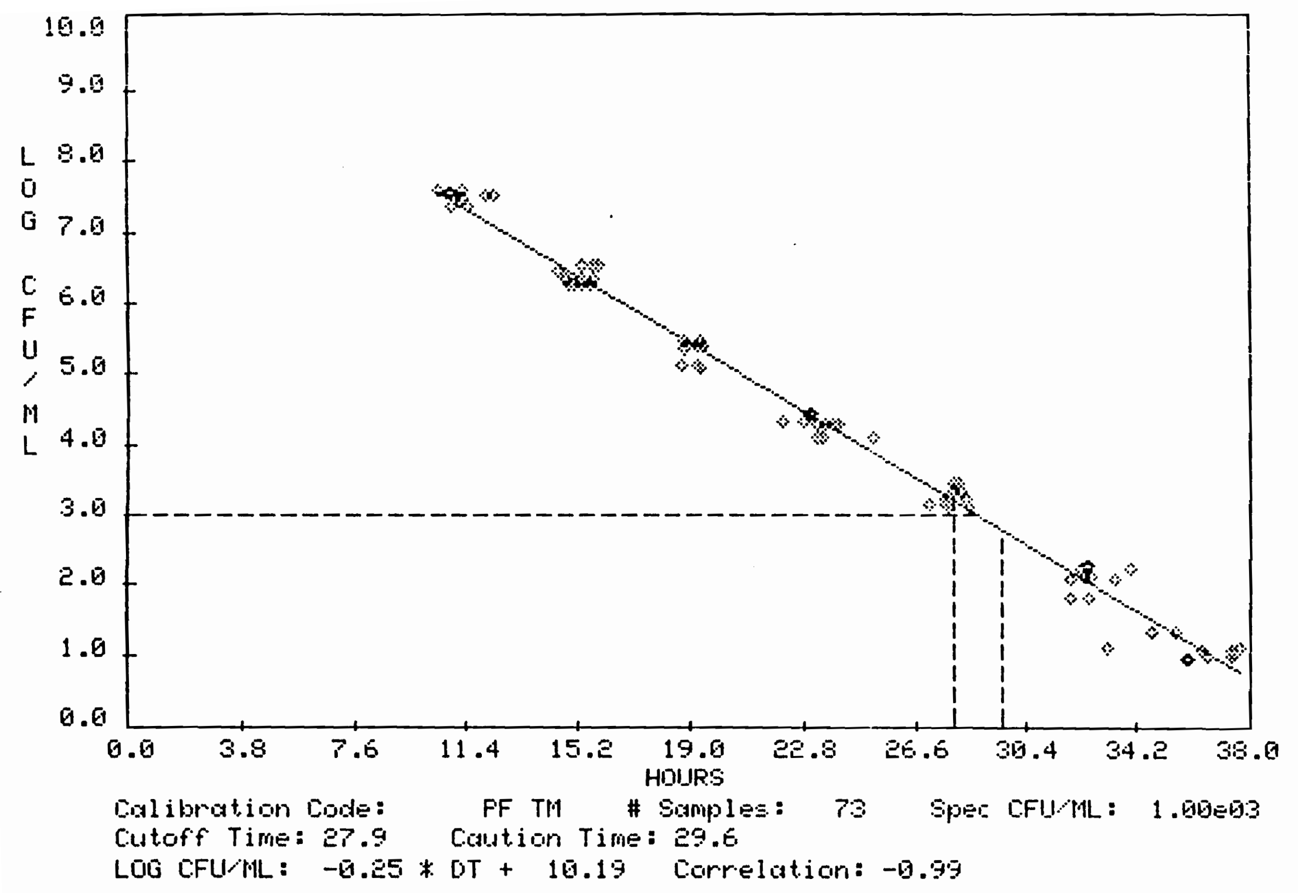


Figure 2 - Standard curve for Pseudomonas fluorescens enumeration by impedance microbiology

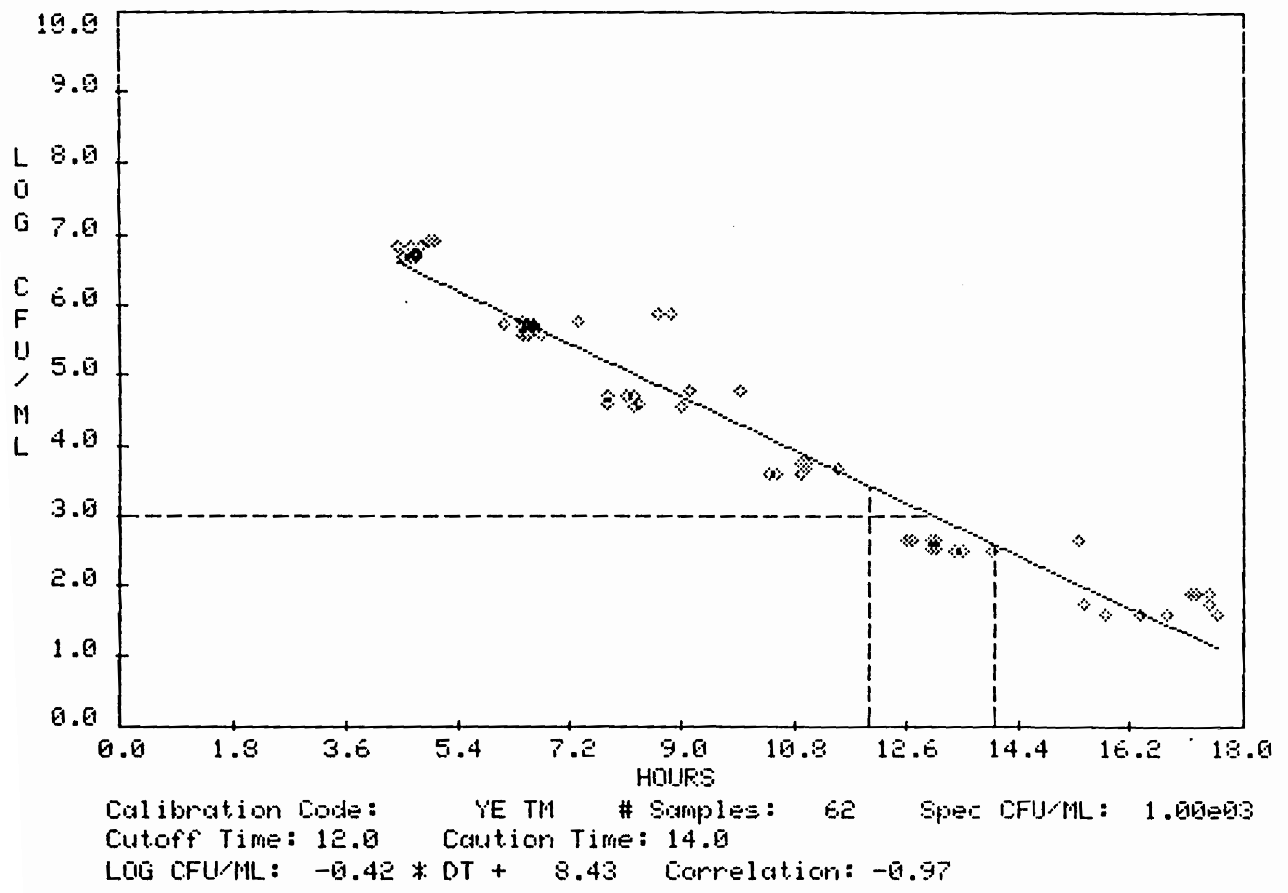


Figure 3 - Standard curve for Yersinia enterocolitica enumeration by impedance microbiology

the surface of the equipment preventing subsequent contamination. The individual parts of a cleaning system function together to produce a clean surface with very low levels of bacterial contamination (Figures 7-21, Appendix A)). The purpose of the cleaning system is not to sterilize the surface, but to reduce the bacterial load to hygienic levels where future product is not at risk. The inability of the cleaning system to remove or destroy Y. enterocolitica could be a function of its ability to tightly adhere to the surface or the type and amount of glycocalyx produced by the organism. This is a serious concern to the dairy industry since Y. enterocolitica is a psychrotrophic pathogen.

All sanitizer solutions applied in the pipeline system were equally effective (Tables 10, 12, and 14). The reduction of P. fluorescens ranged from 3.09 log_{10} cells/cm^2 with acid anionic sanitizer on rubber to 0.96 log_{10} cells/cm^2 with chlorine on Teflon® (Table 10). The sanitizers were least effective toward Y. enterocolitica with a reduction in cell numbers ranging from 0.59 log_{10} cells/cm^2 with acid anionic on rubber to 0.02 log_{10} cells/cm^2 with chlorine on Teflon® (Table 12). The variation in the effectiveness of each sanitizer toward the three bacteria demonstrated the complex nature of biofilms. At this point, the types of exopolysaccharides that are being produced by each organism are still unknown. Each organism may produce a unique polysaccharides and the cells may produce more than one type. Research in this area needs to be conducted in order to fully elucidate biofilm behavior.

Table 8 - Initial attachment of pure culture bacteria to rubber and Teflon in a simulated milk pipeline system (Log cells/cm^2 ; n = 10)

	RUBBER	TEFLON
P. fluorescens	6.27[a]	6.19[a]
Y. enterocolitica	5.06[b]	4.93[b]
B. cereus	3.37[c]	2.85[d]

* Means with different letters are significantly different ($P < 0.05$)

Table 9 - Log kill of attached Pseudomonas fluorescens exposed to 200 ppm chlorine in a simulated milk pipeline system (Log cells/cm^2; n = 2).

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	6.20	5.86		
PRE-RINSE	4.76	4.49	1.44 [a]	1.37 [a]
CLEANER	3.46	2.49	1.30 [b]	2.00 [b]
POST-RINSE	1.65	1.10	1.81 [c]	1.39 [c]
SANITIZER (Chlorine)	0.0	0.14	1.65 [d]	0.96 [d]
TOTAL			6.20 [e]	5.72 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 10 - Sanitizer reduction of Pseudomonas fluorescens attached to rubber and teflon in a simulated milk pipeline system (Log cells/cm^2 ; n = 2)

SANITIZER	RUBBER (SURVIVORS)	TEFLON (SURVIVORS)
CHLORINE	1.65[b] (0.0)	0.96[b] (0.14)
IODOPHOR	2.71[ab] (0.22)	2.90[a] (0.0)
ACID ANIONIC	3.09[a] (0.02)	2.81[a] (0.0)
PERACETIC ACID	2.29[ab] (0.33)	1.91[ab] (0.0)
FATTY ACID	1.92[ab] (0.59)	1.20[b] (1.38)

* Means across each row with different letters are significantly different ($P < 0.05$)

* Means down each column with different letters are significantly different ($P < 0.05$)

Table 11 - Log kill of attached Yersinia enterocolitica exposed to 200 ppm chlorine in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	4.77	5.03		
PRE-RINSE	4.69	4.70	0.08[a]	0.33[a]
CLEANER	3.89	4.20	0.80[b]	0.50[b]
POST-RINSE	3.85	3.97	0.04[c]	0.23[c]
SANITIZER (Chlorine)	3.74	3.95	0.10[d]	0.02[d]
TOTAL			1.02[e]	1.08[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 12 - Sanitizer reduction of Yersinia enterocolitica attached to rubber and teflon in a simulated milk pipeline system (Log cells/cm^2; n = 2)

SANITIZER	RUBBER (SURVIVORS)	TEFLON (SURVIVORS)
CHLORINE	0.10[a] (3.74)	0.02[ab] (3.95)
IODOPHOR	0.34[a] (2.91)	0.34[a] (2.82)
ACID ANIONIC	0.59[a] (3.41)	0.24[a] (3.78)
PERACETIC ACID	0.40[a] (2.95)	0.19[a] (3.00)
FATTY ACID	0.19[a] (3.05)	0.33[a] (2.82)

* Means across each row with different letters are significantly different ($P < 0.05$)

* Means down each column with different letters are significantly different ($P < 0.05$)

Table 13 - Log kill of attached Bacillus cereus exposed to 200 ppm chlorine in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.36	2.34		
PRE-RINSE	3.20	2.19	0.16 [a]	0.16 [a]
CLEANER	2.27	1.84	0.93 [b]	0.36 [b]
POST-RINSE	1.88	1.21	0.40 [c]	0.62 [c]
SANITIZER (Chlorine)	0.63	0.55	1.13 [d]	0.66 [d]
TOTAL			2.62 [e]	1.80 [f]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 14 - Sanitizer reduction of Bacillus cereus attached to rubber and teflon in a simulated milk pipeline system (Log cells/cm^2; n = 2)

SANITIZER	RUBBER (SURVIVORS)	TEFLON (SURVIVORS)
CHLORINE	1.13[a] (0.63)	0.66[a] (0.55)
IODOPHOR	1.21[a] (0.64)	1.01[a] (0.55)
ACID ANIONIC	0.50[a] (0.0)	0.70[a] (0.0)
PERACETIC ACID	0.84[a] (0.94)	0.80[a] (0.80)
FATTY ACID	0.70[a] (0.12)	0.92[a] (0.36)

* Means across each row with different letters are significantly different ($P < 0.05$)

* Means down each column with different letters are significantly different ($P < 0.05$)

IV. Efficacy of Sanitizers Towards Attached Mixed Cultures of Bacteria in a Simulated Milk Pipeline System

Preliminary research was conducted to develop selective media for use in the Bactometer®. Results indicated promising results for a selective medium for L. monocytogenes. Fifteen g/l of lithium chloride was incorporated into listeria enrichment broth (LEB). In addition, oxford antibiotic supplement was included. These modifications provided a selective medium that generated good impedance curves with the inhibition of Yersinia and Pseudomonas. In order to broaden the range of usefulness, the test temperature was decrease from 37 to 25°C. This effectively suppressed the growth of Staphylococcus aureus. Pseudomonas isolation medium which included peptone, magnesium chloride, potassium sulfate, and glycerol provided an excellent selection medium for P. fluorescens when 8 ppm polymyxin B was added. Attempts to isolate Y. enterocolitica from mixed culture were unsuccessful. Modifications to CIN agar failed to suppress the growth of P. fluorescens. Therefore, it was necessary to use plating techniques to enumerate the three test bacteria in mixed culture. Although P. fluorescens demonstrated growth on CIN agar, a distinction could be made using visual observations. Y. enterocolitica colonies on CIN agar exhibited a characteristic red "bullseye" with a transparent border.

Similar to the results from the pure culture data, the initial attachment of mixed culture bacteria did not differ between rubber and Teflon® (Tables 16, 18,

and 20). Again, this may be due to the fluid flow dynamics in the pipeline system. P. fluorescens attached at significantly higher numbers with 4.58 $\log_{10}$ cells/cm^2 on rubber and 4.32 $\log_{10}$ cells/cm^2 (Table 15). Unlike the pure culture studies, the numbers of attached Y. enterocolitica were effectively reduced in the pipeline system, when in mixed culture. There are several plausible explanations. First, the initial inoculum was at a lower level for each organism in mixed culture (approximately 1/3 of that in pure culture). This could have lead to decreased attachment due to a reduction in surface contact opportunities. Bryers and Characklis (23) stated that the in turbulent flow conditions the rate of overall bacterial adhesion increases with the biomass concentration.

In addition, the behavior of Yersinia in mixed culture may be altered in the presence of other organisms. Costerton and Scott (38) stated that when several bacterial species are present, patterns of adherence are altered. The presence of competing organisms may influence the type and amount of exopolysaccharide production and the growth of the bacterium itself may be restrained. In this study, other cells in the system could have attached preferentially to the surfaces thus preventing firm adhesion of Yersinia. Y. enterocolitica may adhere to the primary layer of attached Pseudomonas or Bacillus. Since these cells are more easily removed the Yersinia cells are also washed away or destroyed. Speers, et al. (207) showed that cells did not accumulate after repeated inoculation and washing steps. They suggest that the secondary layer of cells, those not directly attached to

the surface, were more easily removed.

For the most part, there were no significant differences in sanitizer efficacy toward the attached bacteria (Tables 17, 19, 21). For each bacteria/gasket combination, the sanitizers were equally effective. The only exception was P. fluorescens attached to Teflon® (Table 17). Chlorine was shown to be statistically different from the iodophor and acid anionic sanitizer, but this does not mean that chlorine was ineffective, instead it reflects the lack of survivors from the post-rinse step (Figures 22-30, Appendix A).

With both pure and mixed cultures, the sanitizer applied after the post-rinse had little effect on the overall result. With few exceptions, the steps of the cleaning system were all significant. The entire cleaning regime reduces bacterial numbers to acceptable levels; the sanitizer did not accomplish the entire goal alone (Tables 34-39, Appendix B). Studies have shown that bacteria surrounded by exopolysaccharide material have increased resistance to antimicrobial agents. LeChevallier, et al. (127) illustrated the increased resistance of attached bacteria with studies involving Klebsiella pneumoniae attached to glass slides. They found that K. pneumoniae exhibited a 150 fold increase in resistance to antimicrobial agents. Frank and Koffi (82) demonstrated that attached L. monocytogenes could survive 20 min of exposure to benzalkonium chloride (100, 400, and 800 ppm) and acid anionic sanitizer (200 and 400 ppm). In contrast, planktonic cells were reduced to undetectable levels after 30 sec of exposure to the lowest

Table 15 - Initial attachment of mixed culture bacteria to rubber and teflon in a simulated milk pipeline system (Log cells/cm^2; n = 10)

	RUBBER	TEFLON
P. fluorescens	4.58[a]	4.32[a]
Y. enterocolitica	3.17[b]	2.84[b]
B. cereus	3.31[b]	2.90[b]

* Means across each row with different letters are signiifcantly different (P < 0.05)

* Means down each column with different letters are signiifcantly different (P < 0.05)

Table 16 - Log kill of attached Pseudomonas fluorescens from mixed culture exposed to 25 ppm iodophor in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	4.83	4.38		
PRE-RINSE	2.80	3.85	2.03 [a]	0.53 [b]
CLEANER	2.48	2.47	0.32 [b]	1.38 [c]
POST-RINSE	1.68	1.55	0.8 [c]	0.92 [c]
SANITIZER (Iodophor)	0.0	0.0	1.68 [d]	1.55 [d]
TOTAL			4.83 [e]	4.38 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 17 - Sanitizer reduction of Pseudomonas fluorescens from mixed culture attached to rubber and teflon in a simulated milk pipeline sytem (Log cells/cm ^{2}; n = 2)

SANITIZER	RUBBER (SURVIVORS)	TEFLON (SURVIVORS)
CHLORINE	0.98 [a] (0.0)	NA [1]
IODOPHOR	1.68 [a] (0.0)	1.55 [a] (0.0)
ACID ANIONIC	1.36 [a] (0.0)	1.51 [a] (0.0)

* Means across each row with different letters are significantly different (P < 0.05)

* Means down each column with different letters are significantly different (P < 0.05)

1- No survivors after the post-rinse step

Table 18 - Log kill of attached Yersinia enterocolitica from mixed culture exposed to 25 ppm iodophor in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.26	2.37		
PRE-RINSE	2.28	1.40	0.98 [a]	0.97 [a]
CLEANER	1.34	1.25	0.94 [b]	0.15 [b]
POST-RINSE	0	0	1.34 [c]	1.25 [c]
SANITIZER (Iodophor)	NA [1]	NA [1]	NA [1]	NA [1]
TOTAL			3.26 [d]	2.37 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

1 - No survivors after the post-rinse step

Table 19 - Sanitizer reduction of Yersinia enterocolitica from mixed culture attached to rubber and teflon in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

SANITIZER	RUBBER (SURVIVORS)	TEFLON (SURVIVORS)
CHLORINE	1.13[a] (0.58)	NA[1]
IODOPHOR	NA[1]	NA[1]
ACID ANIONIC	0.58[a] (0.0)	NA[1]

* Means down each column with different letters are significantly different ($P < 0.05$)

1 - No survivors after the post-rinse step

Table 20 - Log kill of attached Bacillus cereus from mixed culture exposed to 25 ppm iodophor in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.26	2.55		
PRE-RINSE	2.27	1.86	0.99 [a]	0.68 [a]
CLEANER	1.37	1.49	0.90 [b]	0.37 [b]
POST-RINSE	0.85	0.0	0.52 [c]	1.49 [d]
SANITIZER (Iodophor)	0.0	NA [1]	0.85 [d]	NA [1]
TOTAL			3.26 [e]	2.55 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

1 - No survivors after the post-rinse step

Table 21 - Sanitizer reduction of Bacillus cereus from mixed culture attached to rubber and teflon in a simulated milk pipeline system (Log cells/cm^2 ; n = 2)

SANITIZER	RUBBER (SURVIVORS)	TEFLON (SURVIVORS)
CHLORINE	1.23[a] (0.0)	0.50[a] (0.0)
IODOPHOR	0.85[a] (0.0)	NA[1]
ACID ANIONIC	1.25[a] (0.0)	1.26[a] (0.0)

* Means across each row with different letters are significantly different (P < 0.05)

* Means down each column with different letters are significantly different (P < 0.05)

1 - No survivors from post-rinse step

concentration of the sanitizer. Lee and Frank (129) observed that 8 day adherent cells of L. monocytogenes were 100 times more resistant than 4 day cells when exposed to 200 ppm hypochlorite for 30 sec.

This part of the study showed great variation in the number of survivors at the end of the post-rinse step. With Y. enterocolitica attached to rubber and exposed to 25 ppm iodophor, there were no survivors after the post-rinse step; however, the chlorine and acid anionic sanitizer tests show 1.13 $\log_{10}$ cells/cm^2 and 0.58 $\log_{10}$ cells/cm^2, respectively (Table 19). All tests were exactly the same up to the sanitizer step. These differences are difficult to explain, but may reflect the weak attractive forces present in the mixed culture system.

CIP systems represent a possible source of concern for the dairy industry. If these systems are not capable of reducing the adherent bacterial population, there is the potential for contamination of future product as it passes over the food contact surface. Maxcy (148) states that CIP systems are especially troublesome because the equipment is not accessible for visual inspection. He emphasizes that adherent microcolonies are likely to be centralized in trouble spots, rather than spread throughout the system. Gaskets materials are particularly susceptible to bacterial attachment. Usually, they are positioned at bends or joints in the pipeline, where the flow of milk tends to slow down.

Czechowski (44) demonstrated that many environmental psychrotrophic bacteria adhere easily to surfaces used in food processing system, such as buna-N

rubber and Teflon® gaskets, and stainless steel. By collecting gasket materials from dairy pipeline systems, he found that gasket materials left in place for extended periods of time become heavily contaminated (46). This presents product quality and health concern since the surfaces of processing, transport, and storage equipment could serve as major sources of contamination. Attached bacteria continue to grow and multiply within the biomatrix. Once attached to the surface, bacteria have a better chance of surviving the cleaning procedures. These cells may detach on their own or be physically removed as product is returned to the pipeline (56, 131). The results of this study showed that the cleaning system used in dairy processing plants is capable of removing adherent cells. Most processors place too much emphasis on the sanitizing step when in reality it is just a small part of the whole removal process. Mixed cultures of bacteria interact with each other during biofilm formation. Studies done with pure cultures in vitro have painted a dismal picture of sanitizer efficacy towards attached bacteria. The situation does not look so desperate when the other parts of the cleaning system are factored in the equation. Rigorous daily cleaning and sanitizing is an effective weapon against biofilm development. The results of this study should make dairies aware that constant attention to detail is necessary. Even if everything is done properly, dairies should maintain a strict schedule of gasket replacement because in time the biofilms will form.

CONCLUSIONS

Bacterial attachment can pose serious quality and health hazard problems in the dairy processing industry. Within the biomatrix, the bacteria are continually growing, multiplying, and being released into the surrounding environment. This could be an important source of product contamination. Since both pathogenic and spoilage bacteria have demonstrated a proclivity for attachment to inert surfaces, their presence should not be overlooked. In this study, bacteria attached to both buna-N rubber and Teflon® with equal efficiency in a pipeline system. The surface effects were diminished when conditions of turbulent flow were present.

Milk provides an excellent growth medium for bacteria. Psychrotrophic pathogenic bacteria, such as Yersinia enterocolitica and Listeria monocytogenes, may proliferate during storage periods to hazardous levels. As a result, the threat to public health is substantial because milk and milk products generally receive little treatment prior to consumption.

Impedance microbiology remains the most practical method of enumeration, since it requires no treatment for the gasket materials. In order to continue mixed culture study, more research is needed on selective media for the Bactometer®. The modified listeria enrichment broth and Pseudomonas isolation medium were effective in suppressing the growth of other species. The blending technique used in this study provided an alternate, effective method of

enumeration.

Prevention is the key to avoiding biofilm development. Once a biofilm is firmly established, cleaning and sanitizing becomes much more difficult. Adherent microcolonies can be eliminated or reduced with the proper cleaning system. Cleaning systems should be maintained at optimal levels in order to function effectively. This study demonstrates the effectiveness of a cleaning system in a simulated milk pipeline.

Results of this study show that bacteria are not likely to survive the cleaning procedures when the proper concentration of cleaner and sanitizer are used at the appropriate temperature, with the exception of Yersinia enterocolitica which demonstrated an increased resistance in pure culture. The entire cleaning/sanitizing regime must be examined in order to help prevent a biofilm build-up. Results of this study indicate that bacteria within a biofilm exhibit different characteristics when in pure and mixed culture. The resistance patterns become altered when more than one species is present. Conclusions drawn from data on pure culture testing could be misinterpreted, since bacteria in nature tend to interact with each other and their surroundings in unique ways.

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APPENDIX A

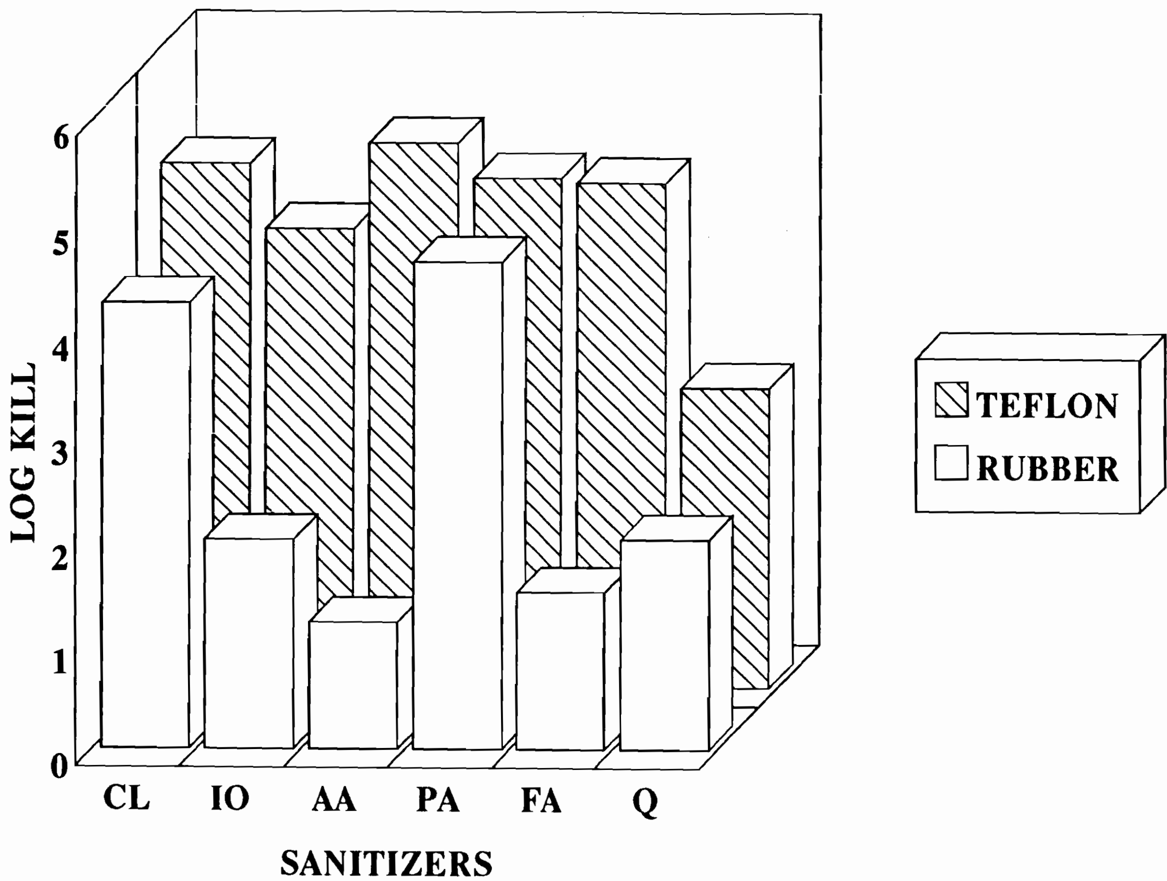

Figure 4 - Log kill of Pseudomonas fluorescens in mixed culture after 30 s exposure to sanitizer

CL = Chlorine; IO = Iodophor; AA = Acid Anionic;
PA = Peracetic Acid; FA = Fatty Acidd; Q= QUAT

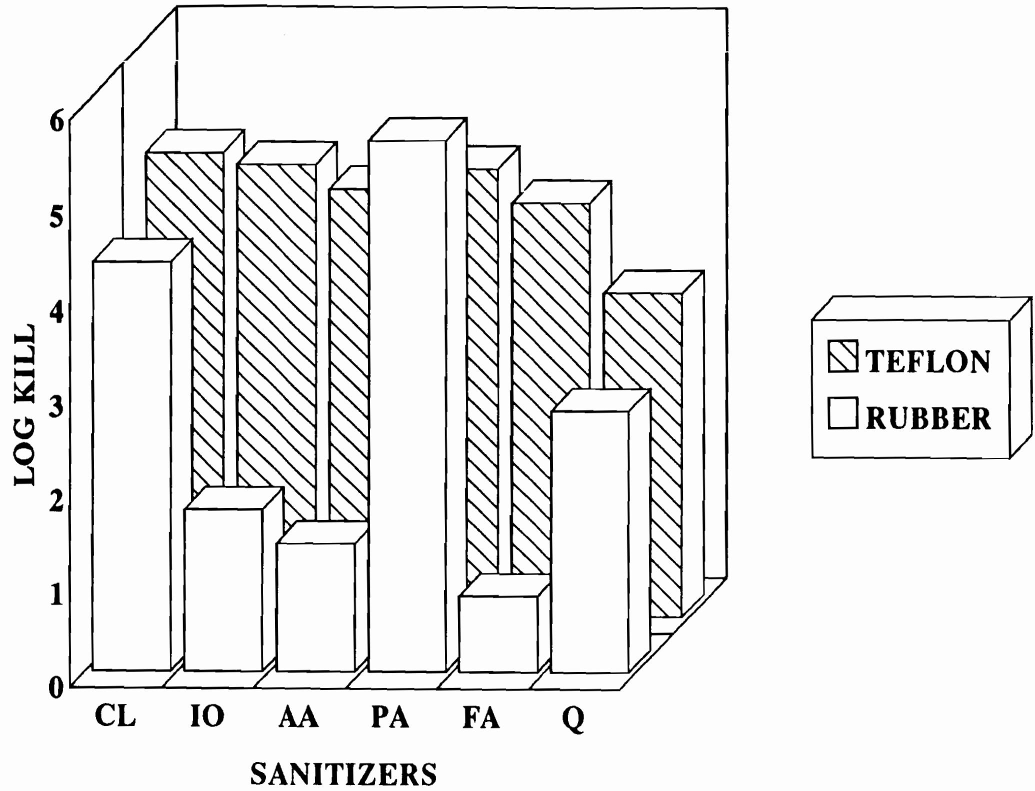

Figure 5 - Log kill of Yersinia enterocolitica in mixed culture after 30 s exposure to sanitizer

CL = Chlorine; IO = Iodophor; AA = Acid Anionic
PA = Peracetic Acid; FA = Fatty Acid; Q = QUAT

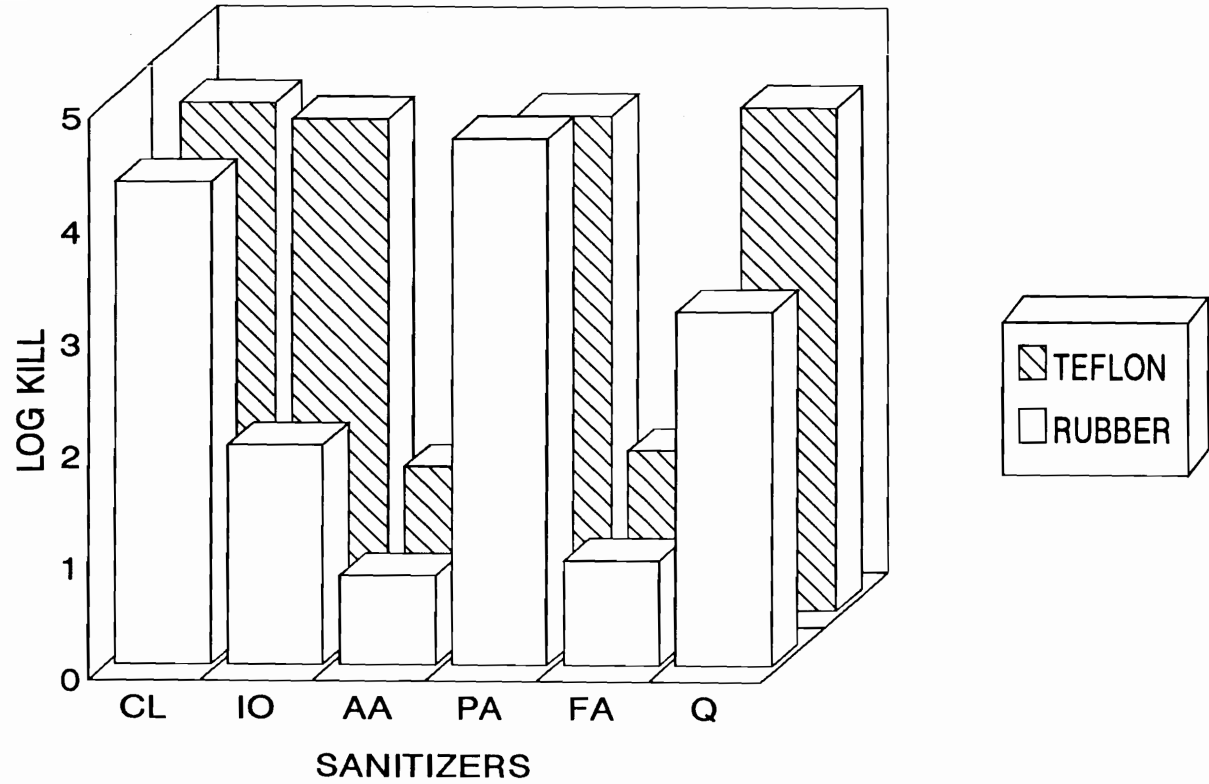

Figure 6 - Log kill of Listeria monocytogenes in mixed culture after 30 s exposure to sanitizer

CL = Chlorine; IO = Iodophor; AA = Acid Anionic; PA = Peracetic Acid; FA = Fatty Acid; Q = QUAT

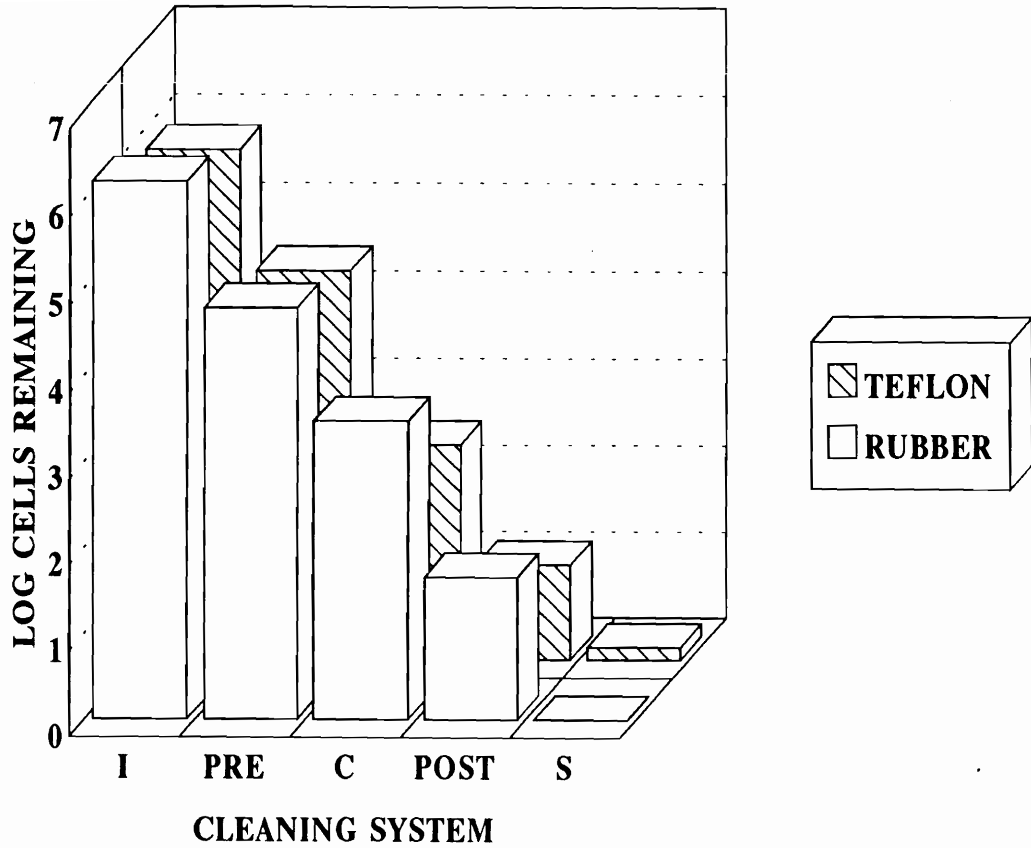

Figure 7 - Log number of remaining attached Pseudomonas fluorescens after exposure to 200 ppm chlorine in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

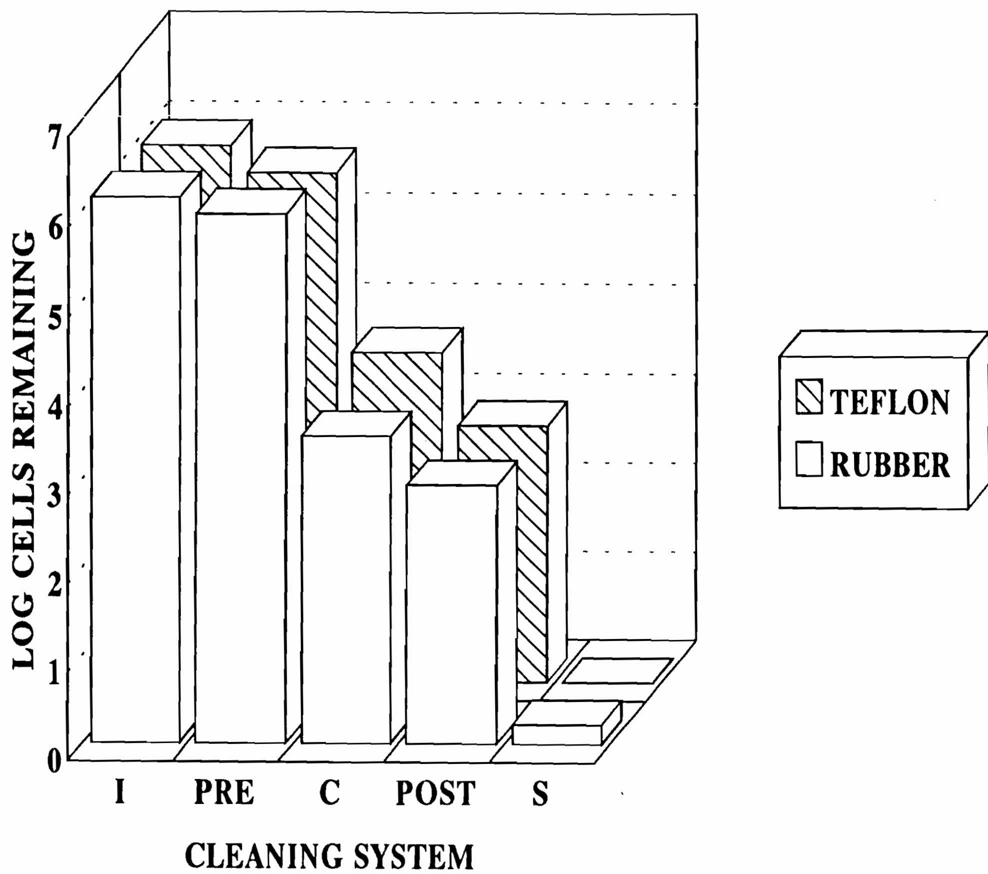

Figure 8 - Log number of remaining attached Pseudomonas fluorescens after exposure to 25 ppm iodophor in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

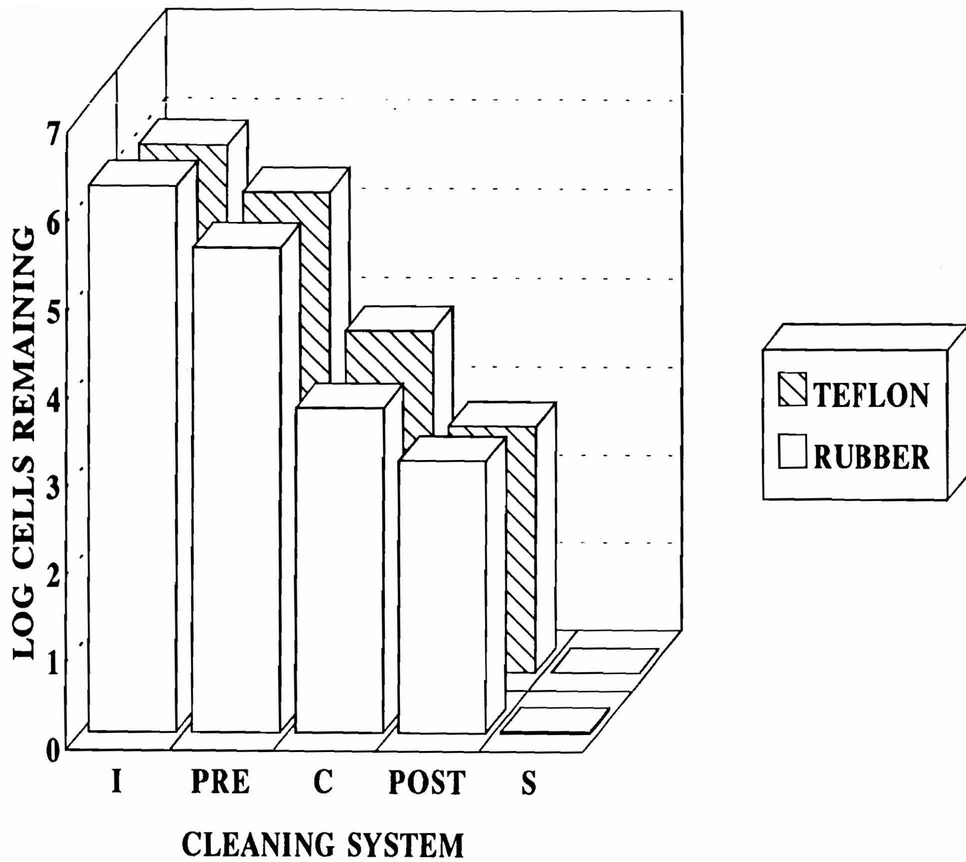

Figure 9 - Log number of remaining attached Pseudomonas fluorescens after exposure to 200 ppm acid anionic sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

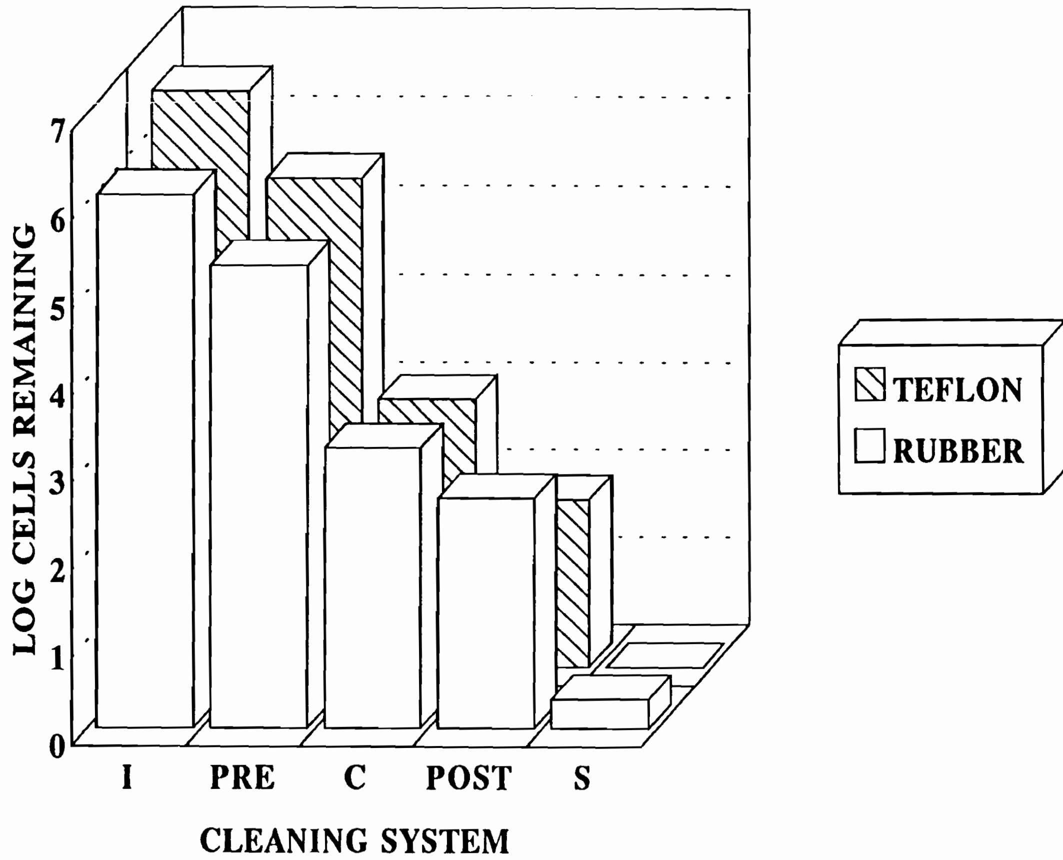

Figure 10 - Log number of remaining attached Pseudomonas fluorescens after exposure to 200 ppm peracetic acid sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

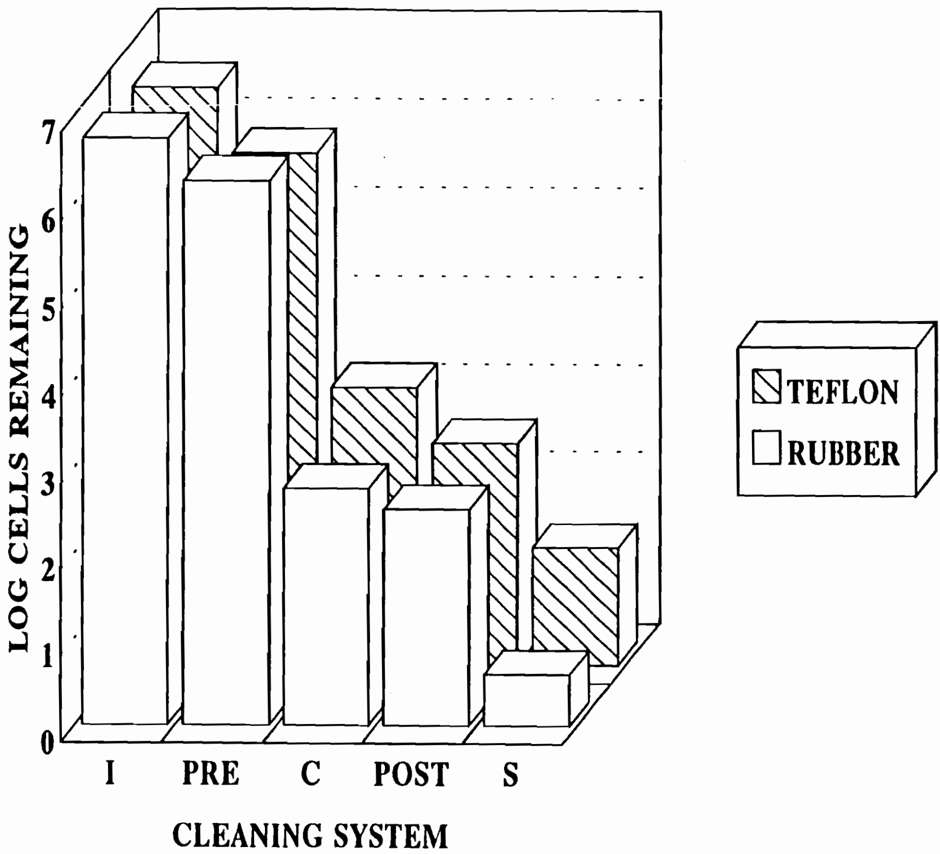

Figure 11 - Log number of remaining attached Pseudomonas fluorescens after exposure to 200 ppm fatty acid sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

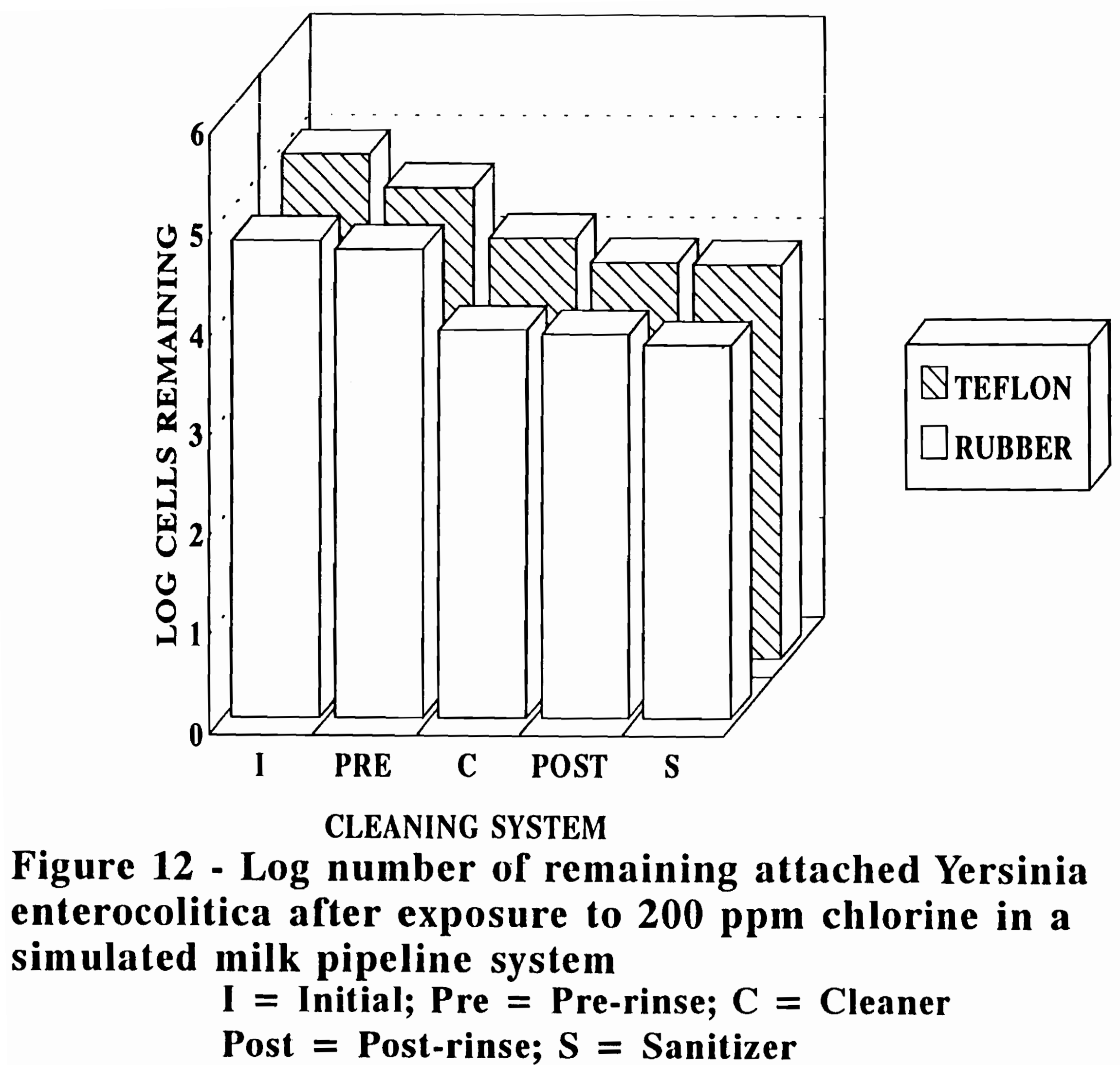

Figure 12 - Log number of remaining attached Yersinia enterocolitica after exposure to 200 ppm chlorine in a simulated milk pipeline system
I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

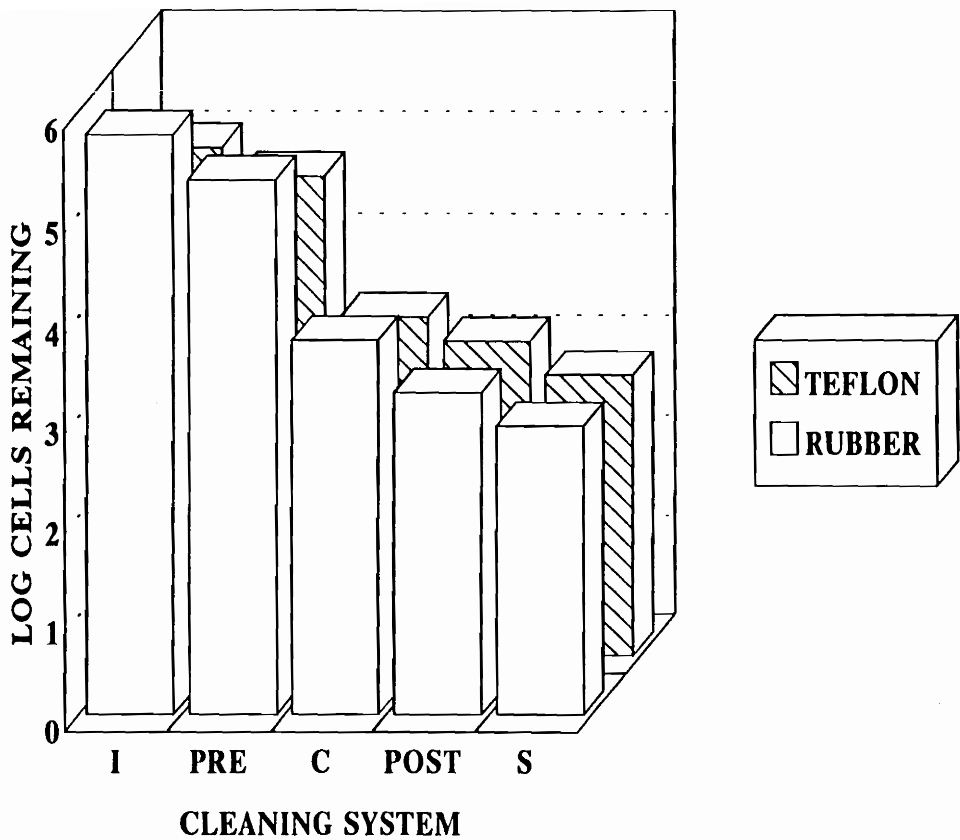

Figure 13 - Log number of remaining attached Yersinia enterocolitica after exposure to 25 ppm iodophor in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

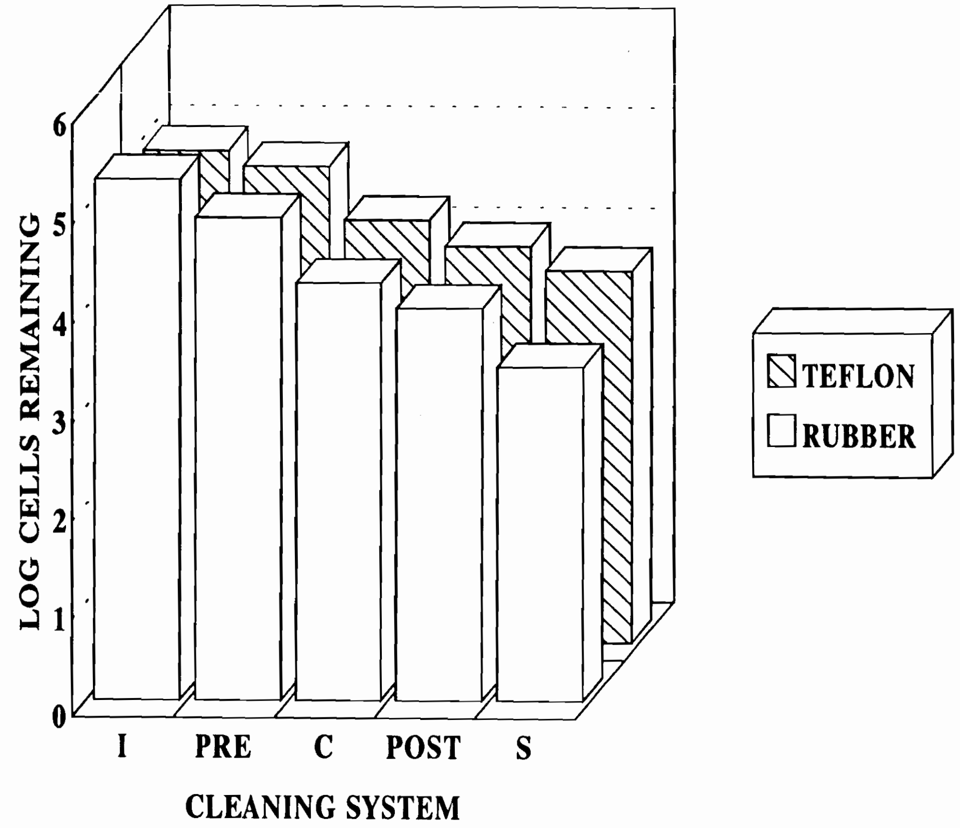

Figure 14 - Log number of remaining attached Yersinia enterocolitica after exposure to 200 ppm acid anionic sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

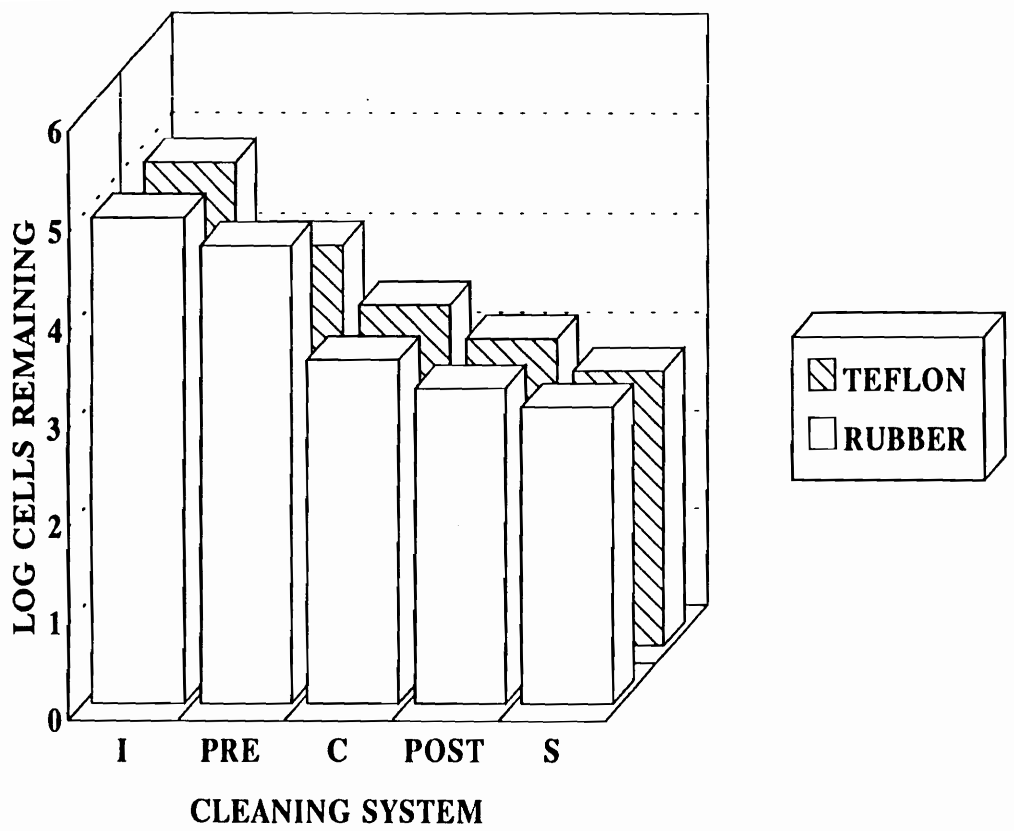

Figure 15 - Log number of remaining attached Yersinia enterocolitica after exposure to 200 ppm peracetic acid sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

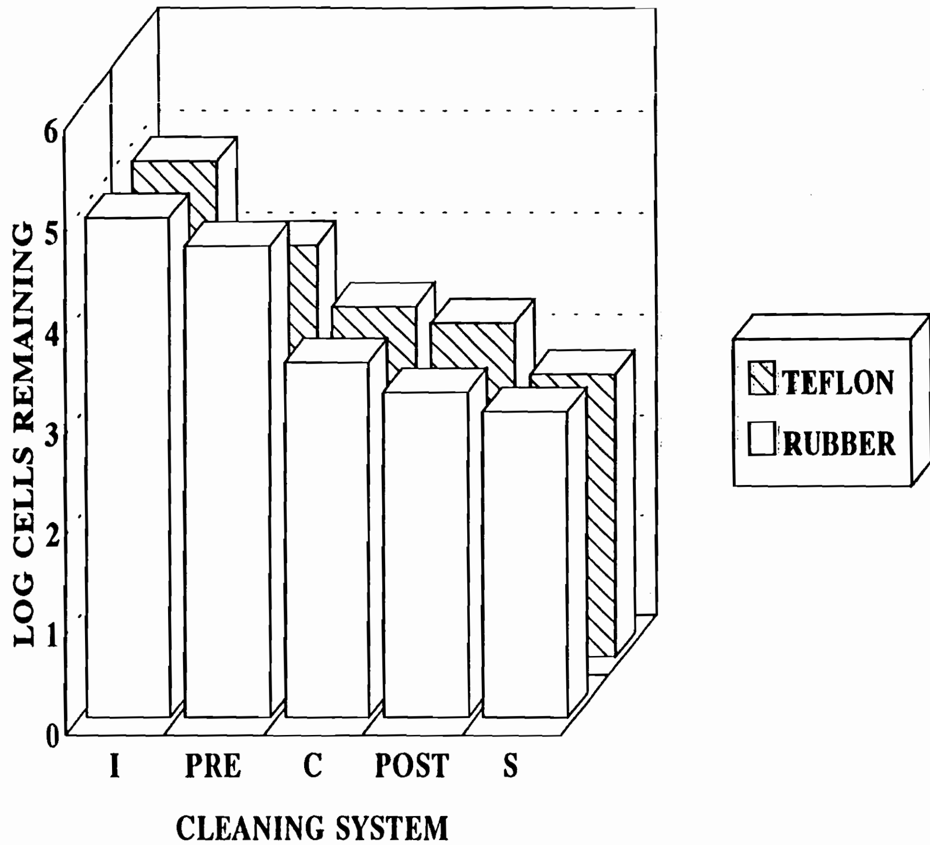

Figure 16 - Log number of remaining attached Yersinia enterocolitica after exposure to 200 ppm fatty acid sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

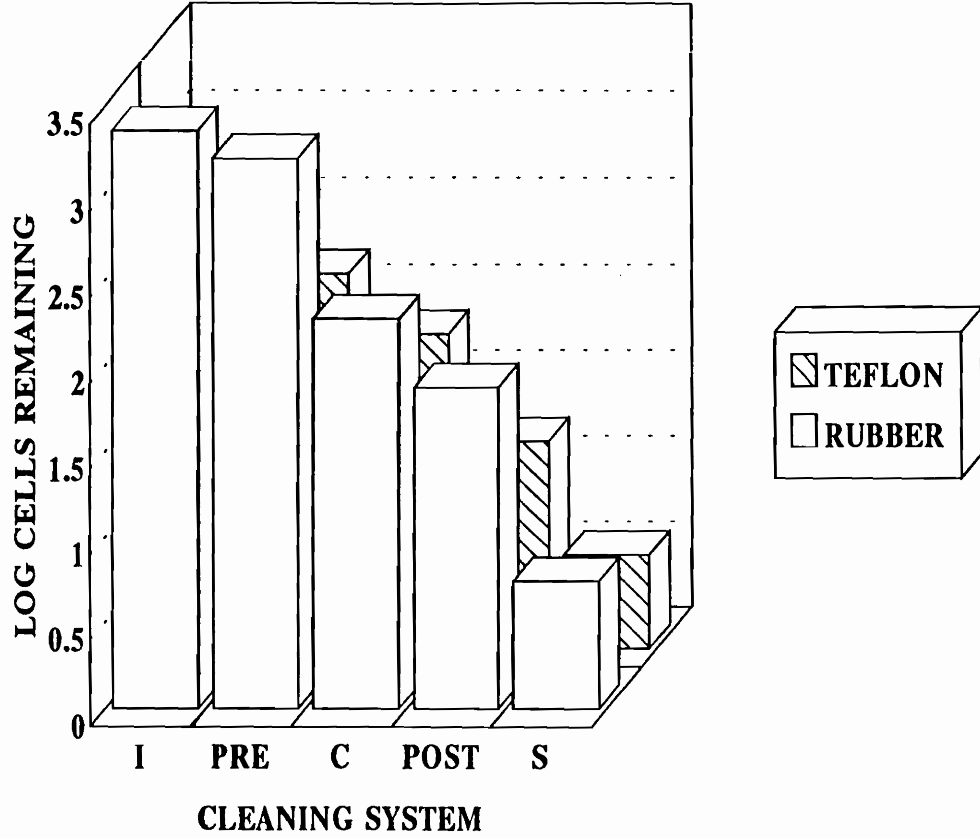

Figure 17 - Log number of remaining attached Bacillus cereus after exposure to 200 ppm chlorine in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

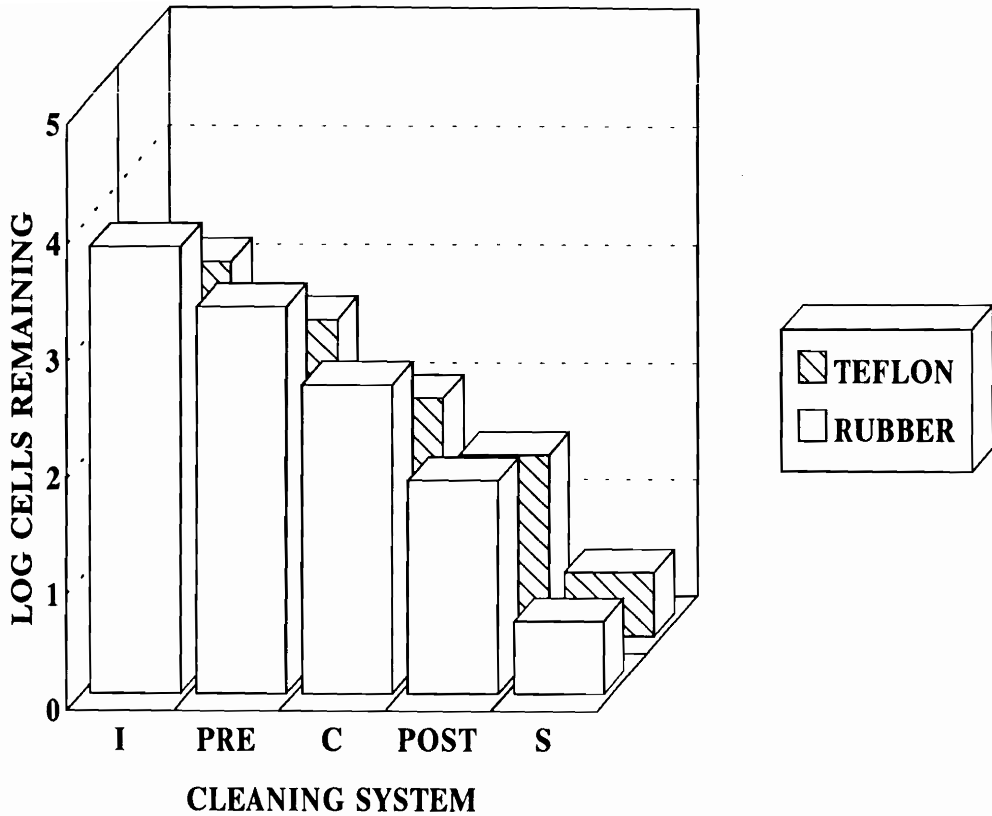

Figure 18 - Log number of remaining attached Bacillus cereus after exposure to 25 ppm iodophor in a simulated milk pipeline sytem

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

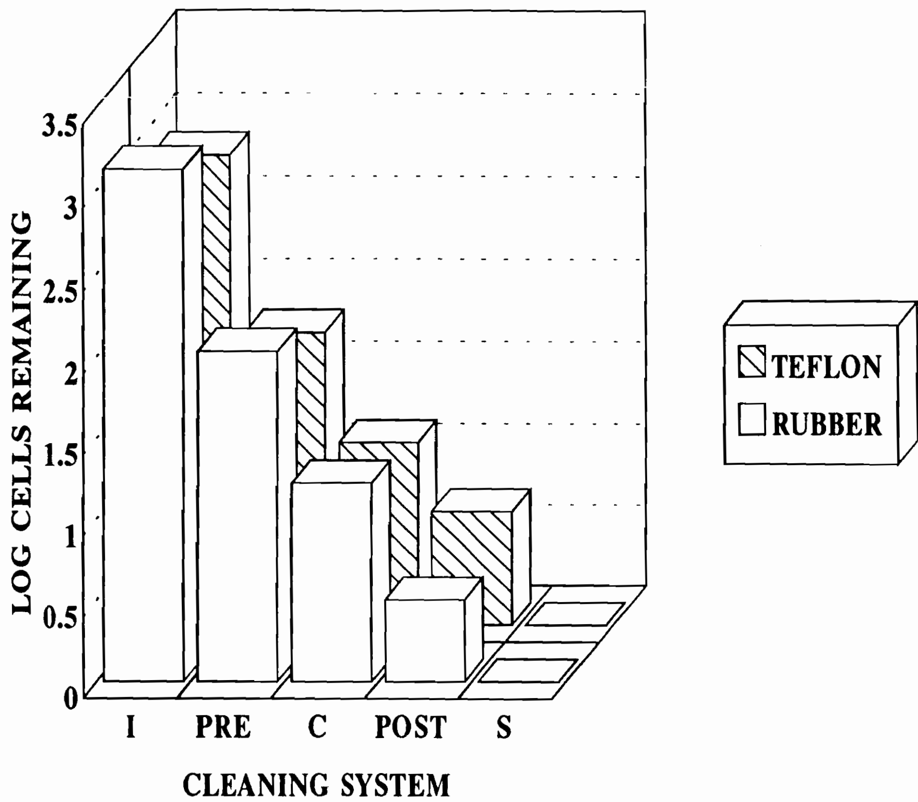

Figure 19 - Log number of remaining attached Bacillus cereus after exposure to 200 ppm acid anionic sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

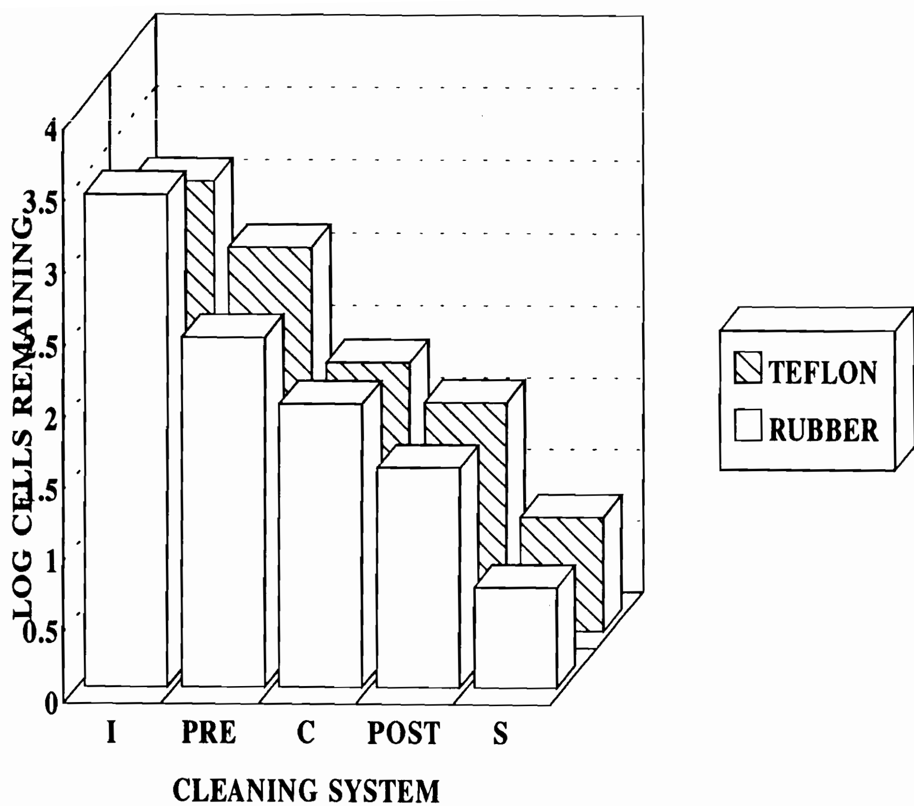

Figure 20 - Log number of remaining attached Bacillus cereus after exposure to 200 ppm peracetic acid sanitizer in a simulated milk pipeline sytem

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

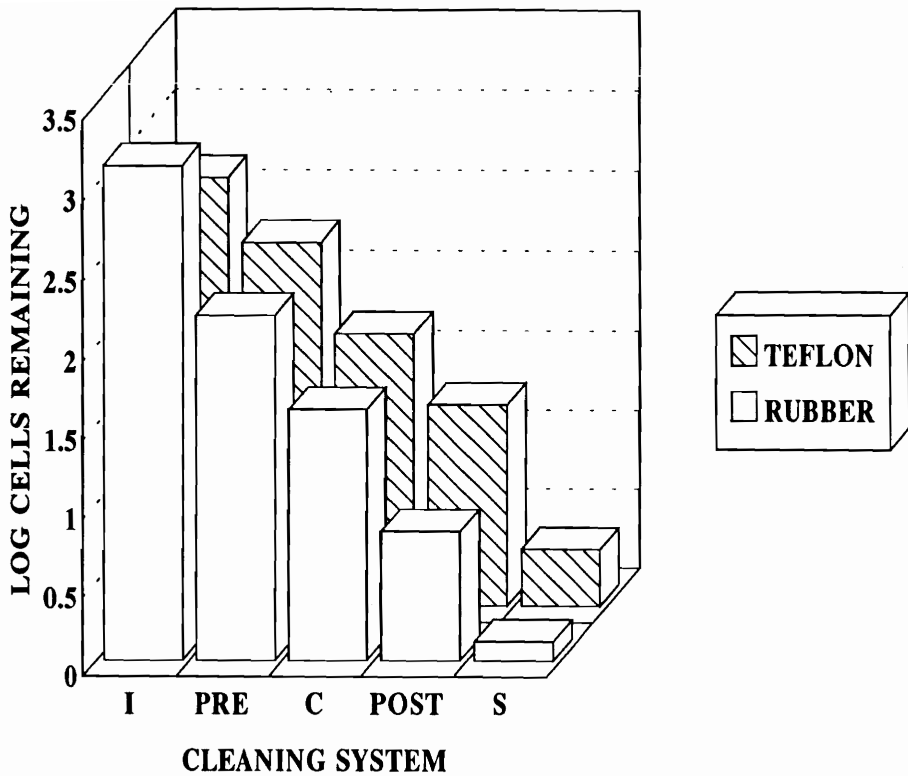

Figure 21 - Log number of remaining attached Bacillus cereus after exposure to 200 ppm fatty acid sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

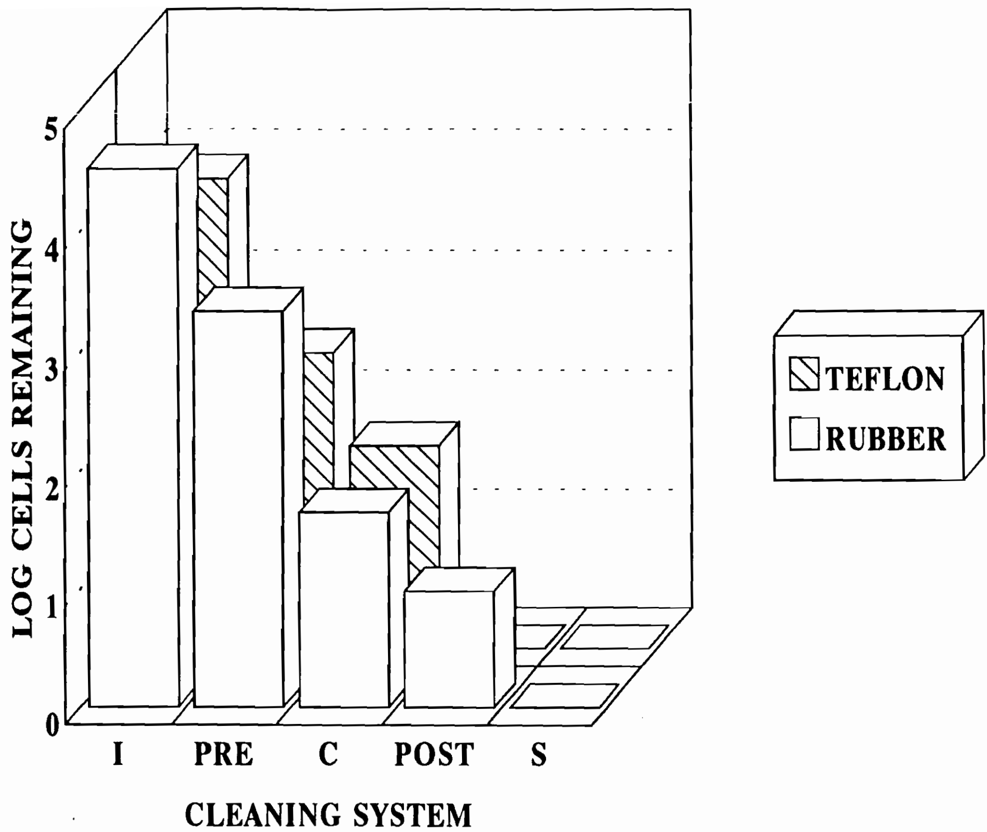

Figure 22 - Log number of remaining attached Pseudomonas fluorescens from mixed culture after exposure to 200 ppm chlorine in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

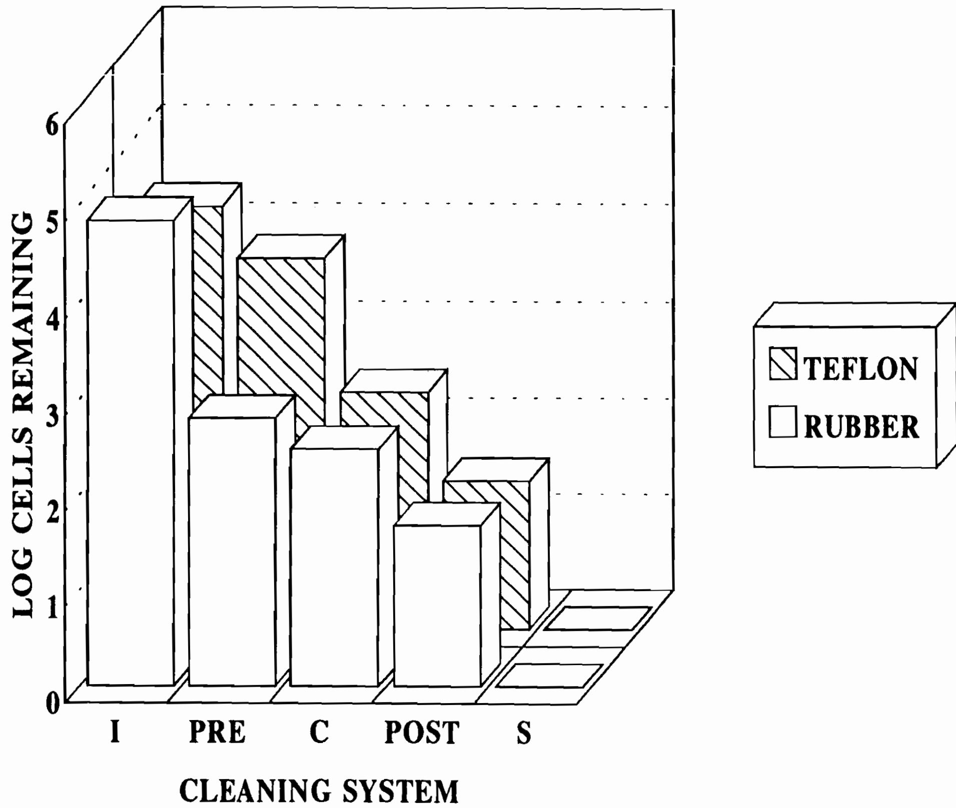

Figure 23 - Log number of remaining attached Pseudomonas fluorescens from mixed culture after exposure to 25 ppm iodophor in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

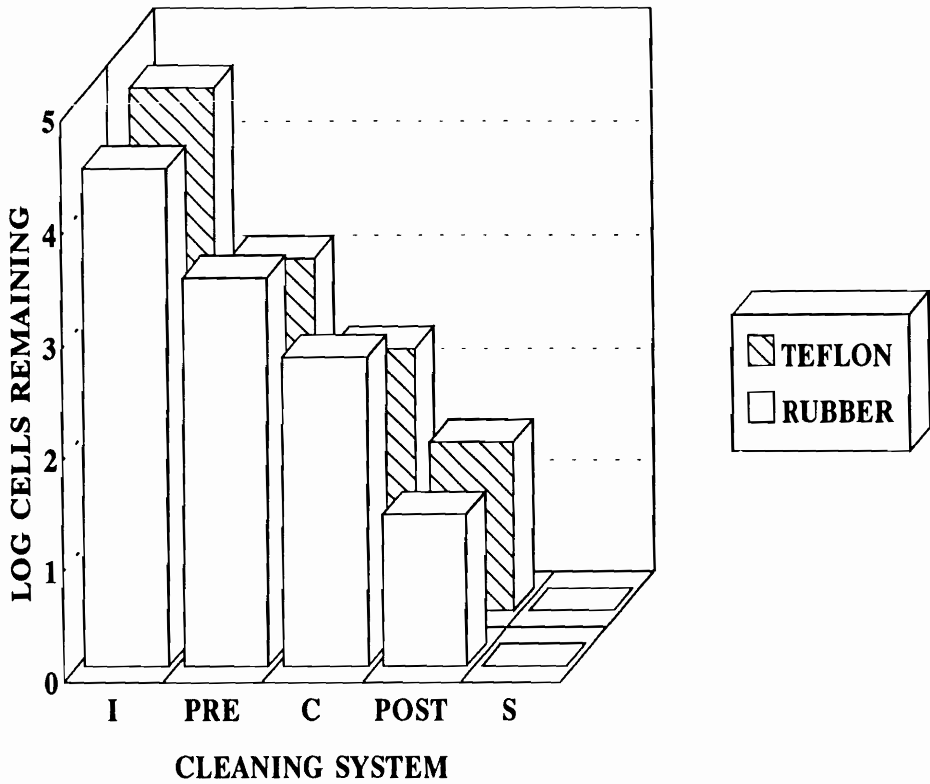

Figure 24 - Log number of remaining attached Pseudomonas fluorescens from mixed culture after exposure to 200 ppm acid anionic sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

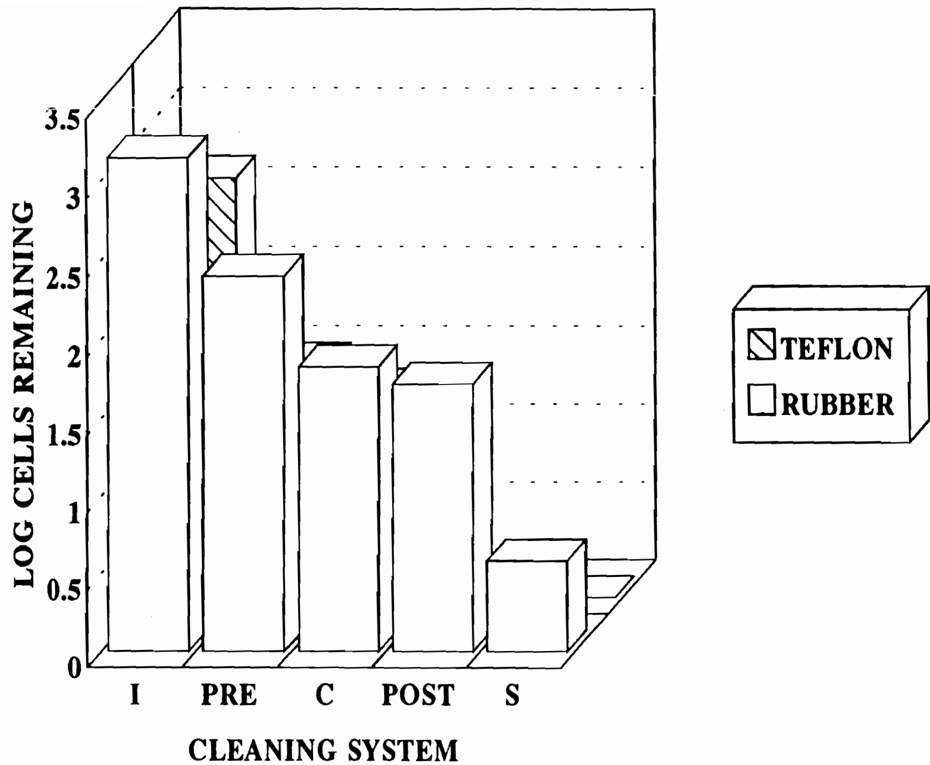

Figure 25 - Log number of remaining attached Yersinia enterocolitica from mixed culture after exposure to 200 ppm chlorine in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

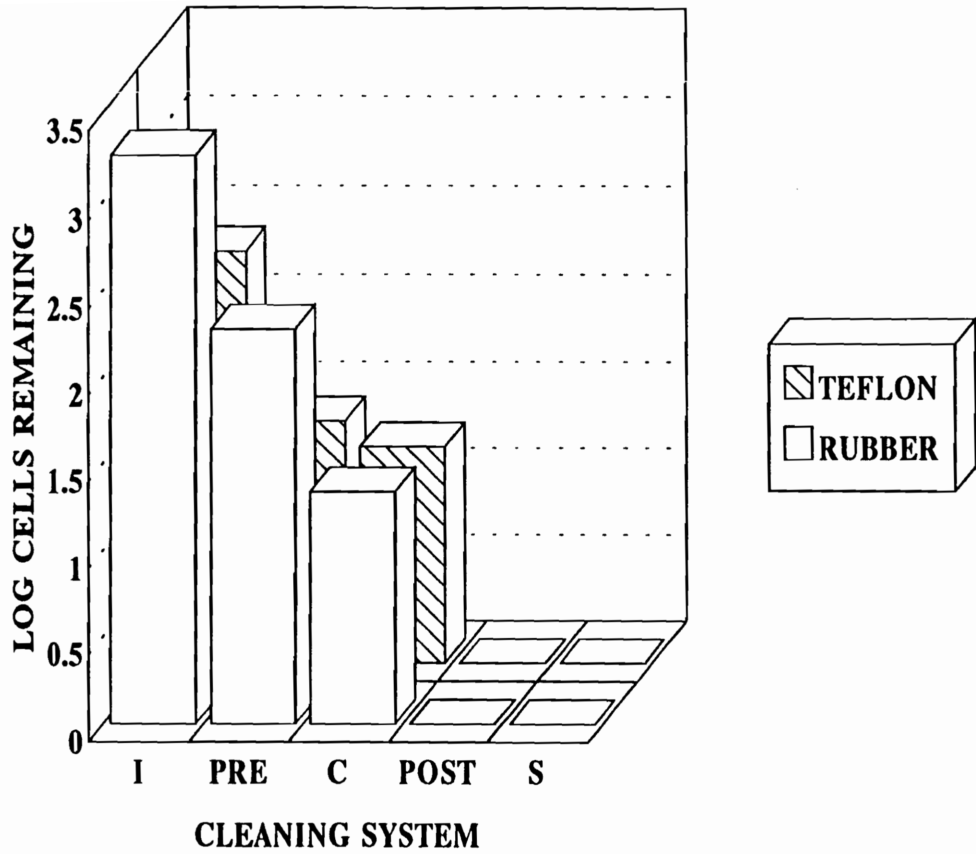

Figure 26 - Log number of remaining attached Yersinia enterocolitica from mixed culture after exposure to 25 ppm iodophor in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

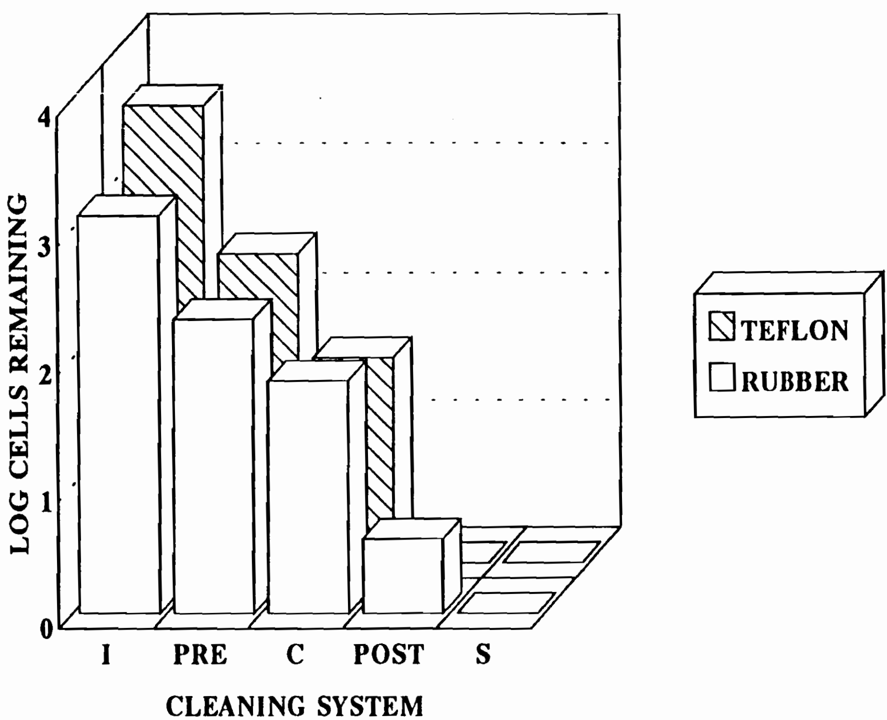

Figure 27 - Log number of remaining attached Yersinia enterocolitica from mixed culture after exposure to 200 ppm acid anionic sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

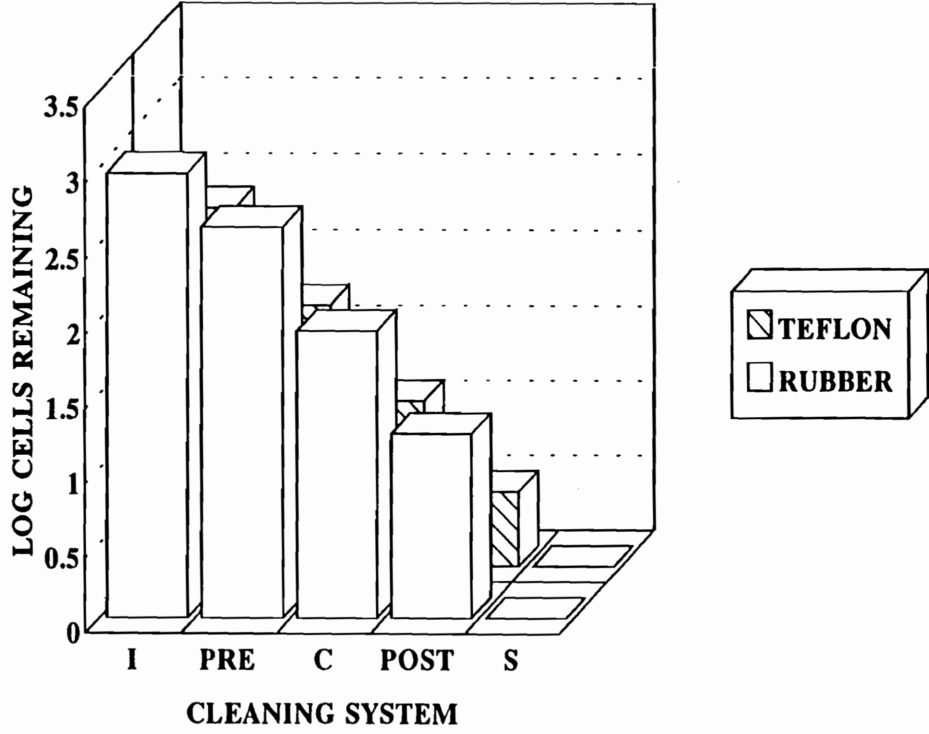

Figure 28 - Log number of remaining attached Bacillus cereus from mixed culture after exposure to 200 ppm chlorine in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

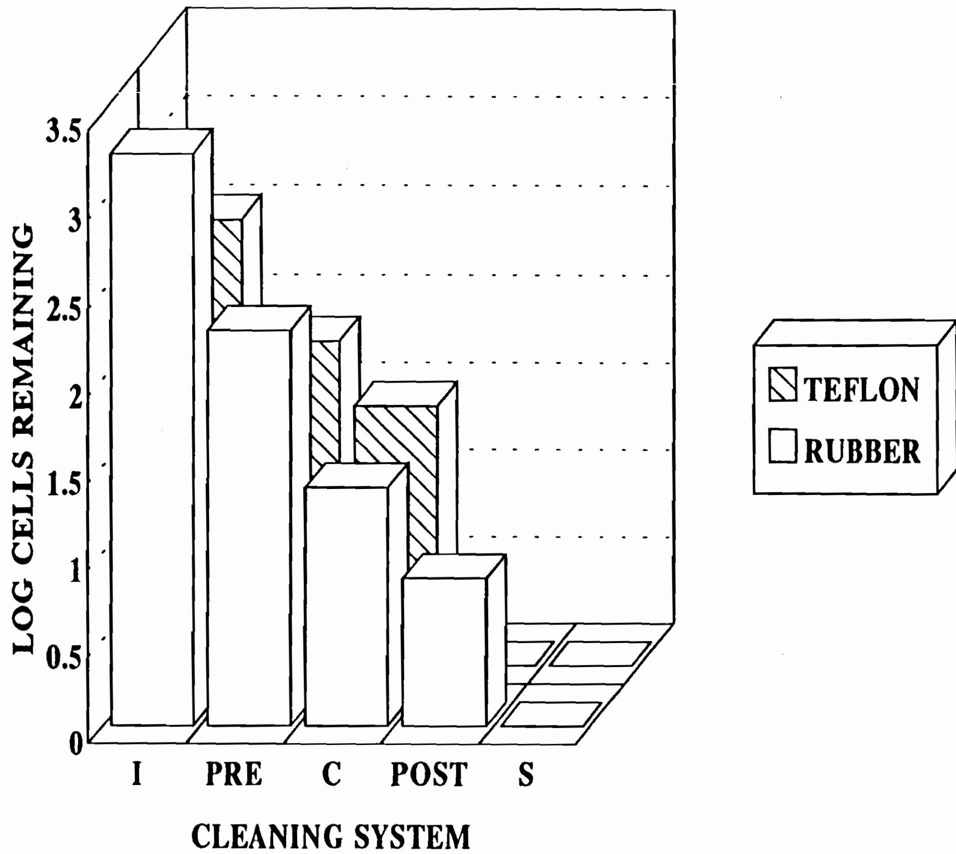

Figure 29 - Log number of remaining attached Bacillus cereus from mixed culture after exposure to 25 ppm iodophor in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

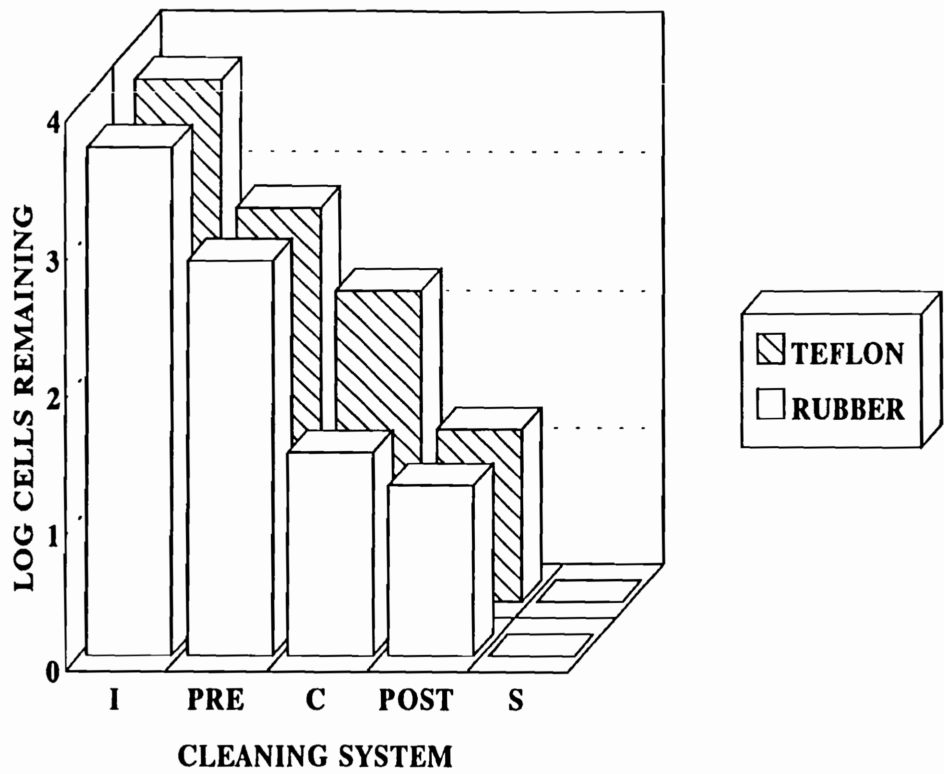

Figure 30 - Log number of remaining attached Bacillus cereus from mixed culture after exposure to 200 ppm acid anionic sanitizer in a simulated milk pipeline system

I = Initial; Pre = Pre-rinse; C = Cleaner
Post = Post-rinse; S = Sanitizer

APPENDIX B

Table 22 - Log kill of attached Pseudomonas fluorescens exposed to 25 ppm iodophor in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	6.13	6.00		
PRE-RINSE	5.94	5.70	0.19 [a]	0.30 [a]
CLEANER	3.48	3.71	2.46 [b]	1.99 [b]
POST-RINSE	2.93	2.90	0.55 [c]	0.81 [c]
SANITIZER (Iodophor)	0.22	0.00	2.71 [d]	2.90 [d]
TOTAL			5.91 [e]	6.00 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 23 - Log kill of attached Pseudomonas fluorescens exposed to 200 ppm acid anionic sanitizer in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	6.20	5.96		
PRE-RINSE	5.51	5.44	0.69 [a]	0.52 [a]
CLEANER	3.71	3.89	1.80 [b]	1.55 [b]
POST-RINSE	3.11	2.81	0.60 [c]	1.07 [c]
SANITIZER (Acid Anionic)	0.02	0.00	3.09 [d]	2.81 [d]
TOTAL			6.18 [e]	5.96 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 24 - Log kill of attached Pseudomonas fluorescens exposed to 200 ppm peracetic acid sanitizer in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	6.01	6.54		
PRE-RINSE	5.29	5.57	0.79 [a]	0.98 [a]
CLEANER	3.20	3.06	2.09 [b]	2.50 [b]
POST-RINSE	2.63	1.91	0.58 [c]	1.15 [c]
SANITIZER (Peracetic Acid)	0.33	0.00	2.29 [d]	1.91 [d]
TOTAL			5.75 [e]	6.54 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 25 - Log kill of attached Pseudomonas fluorescens exposed to 200 ppm fatty acid sanitizer in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	6.73	6.60		
PRE-RINSE	6.25	5.86	0.47 [a]	0.74 [a]
CLEANER	2.75	3.21	3.59 [b]	2.65 [c]
POST-RINSE	2.51	2.58	0.23 [c]	0.64 [c]
SANITIZER (Fatty Acid)	0.59	1.38	1.92 [d]	1.20 [d]
TOTAL			6.21 [e]	5.23 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 26 - Log kill of attached *Yersinia enterocolitica* exposed to 25 ppm iodophor in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	5.78	5.06		
PRE-RINSE	5.34	4.78	0.44[a]	0.28[a]
CLEANER	3.77	3.40	1.57[b]	1.38[b]
POST-RINSE	3.24	3.16	0.53[c]	0.24[c]
SANITIZER (Iodophor)	2.91	2.82	0.34[d]	0.34[d]
TOTAL			2.88[e]	2.24[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 27 - Log kill of attached Yersinia enterocolitica exposed to 200 ppm acid anionic in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	5.28	4.97		
PRE-RINSE	4.90	4.81	0.38 [a]	0.15 [a]
CLEANER	4.25	4.28	0.66 [b]	0.54 [b]
POST-RINSE	4.00	4.02	0.25 [c]	0.26 [c]
SANITIZER (Acid Anionic)	3.41	3.78	0.59 [d]	0.24 [d]
TOTAL			1.88 [e]	1.19 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 28 - Log kill of attached Yersinia enterocolitica exposed to 200 ppm peracetic acid in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	4.52	4.69		
PRE-RINSE	4.06	4.03	0.46 [a]	0.66 [a]
CLEANER	3.39	3.38	0.68 [b]	0.65 [b]
POST-RINSE	3.35	3.22	0.04 [c]	0.18 [c]
SANITIZER (Peracetic Acid)	2.95	3.00	0.40 [d]	0.19 [d]
TOTAL			1.57 [e]	1.69 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 29 - Log kill of attached Yersinia enterocolitica exposed to 200 ppm fatty acid sanitizer in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	4.96	4.92		
PRE-RINSE	4.69	4.10	0.27[a]	0.82[a]
CLEANER	3.53	3.49	1.15[b]	0.61[b]
POST-RINSE	3.24	3.33	0.29[c]	0.16[c]
SANITIZER (Fatty Acid)	3.05	2.82	0.19[d]	0.51[d]
TOTAL			1.91[e]	2.10[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 30 - Log kill of attached Bacillus cereus exposed to 25 ppm iodophor in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.83	3.20		
PRE-RINSE	3.32	2.71	0.51[a]	0.49[a]
CLEANER	2.66	2.05	0.66[b]	0.66[b]
POST-RINSE	1.84	1.56	0.82[c]	0.49[c]
SANITIZER (Iodophor)	0.63	0.55	1.20[d]	1.01[d]
TOTAL			3.20[e]	2.65[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 31 - Log kill of attached Bacillus cereus exposed to 200 ppm acid anionic sanitizer in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.13	2.87		
PRE-RINSE	2.03	1.80	1.10[a]	1.07[a]
CLEANER	1.22	1.12	0.81[b]	0.68[b]
POST-RINSE	0.50	0.70	0.72[c]	0.42[c]
SANITIZER (Acid Anionic)	0.00	0.00	0.50[d]	0.70[d]
TOTAL			3.13[e]	2.87[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 32 - Log kill of attached Bacillus cereus exposed to 200 ppm peracetic acid sanitizer in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.44	3.13		
PRE-RINSE	2.45	2.68	0.98 [a]	0.45 [a]
CLEANER	1.99	1.88	0.46 [b]	0.80 [b]
POST-RINSE	1.49	1.60	0.44 [c]	0.28 [c]
SANITIZER (Peracetic Acid)	0.94	0.80	0.84 [d]	0.80 [d]
TOTAL			2.50 [e]	2.33 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 33 - Log kill of attached Bacillus cereus exposed to 200 ppm fatty acid sanitizer in a simulated milk pipeline system (Log cells/cm ^{2}; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.12	2.69		
PRE-RINSE	2.19	2.29	0.93 [a]	0.40 [a]
CLEANER	1.60	1.72	0.59 [b]	0.57 [b]
POST-RINSE	0.82	1.28	0.77 [c]	0.44 [c]
SANITIZER (Fatty Acid)	0.12	0.36	0.70 [d]	0.92 [d]
TOTAL			3.00 [e]	2.33 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 34 - Log kill of attached Pseudomonas fluorescens from mixed culture exposed to 200 ppm chlorine in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	4.52	3.94		
PRE-RINSE	3.34	2.49	1.18 [a]	1.44 [a]
CLEANER	1.65	1.71	1.69 [b]	0.78 [c]
POST-RINSE	0.98	0	0.66 [c]	1.71 [d]
SANITIZER (Chlorine)	0.0	NA[1]	0.98 [d]	NA[1]
TOTAL			4.52 [e]	3.94 [e]

* Means across each row with different letters are significantly different ($P < 0.05$)

1 - No survivors after the post-rinse step

Table 35 - Log kill of attached Pseudomonas fluorescens from mixed culture exposed to 200 ppm acid anionic sanitizer in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	4.40	4.64		
PRE-RINSE	3.47	3.14	0.92 [a]	1.50 [a]
CLEANER	2.77	2.34	0.70 [b]	0.79 [b]
POST-RINSE	1.36	1.51	1.40 [c]	0.83 [c]
SANITIZER (Acid Anionic)	0.0	0.0	1.36 [d]	1.51 [d]
TOTAL			4.40 [e]	4.64 [e]

* Means across each row with different letter are significantly different ($P < 0.05$)

Table 36 - Log kill of attached *Yersinia enterocolitica* from mixed culture exposed to 200 ppm chlorine in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.15	2.60		
PRE-RINSE	2.40	1.49	0.74[a]	1.10[a]
CLEANER	1.83	1.33	0.57[b]	0.16[ab]
POST-RINSE	1.72	0.0	0.11[c]	1.33[c]
SANITIZER (Chlorine)	0.58	NA[1]	1.13[d]	NA[1]
TOTAL			2.57[e]	2.60[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

1 - No survivors after the post-rinse step

Table 37 - Log kill of attached Yersinia enterocolitica from mixed culture exposed to 200 ppm acid anionic sanitizer in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.11	3.56		
PRE-RINSE	2.31	2.42	0.80[a]	1.13[a]
CLEANER	1.80	1.61	0.51[b]	0.81[ab]
POST-RINSE	0.58	0.0	1.21[c]	1.61[c]
SANITIZER (Acid Anionic)	0.0	NA[1]	0.58[d]	NA[1]
TOTAL			3.11[e]	3.56[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

1 - No survivors after post-rinse step

Table 38 - Log kill of attached Bacillus cereus from mixed culture exposed to 200 ppm chlorine in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	2.96	2.38		
PRE-RINSE	2.61	1.74	0.35[a]	0.64[a]
CLEANER	1.92	1.10	0.68[b]	0.64[b]
POST-RINSE	1.23	0.50	0.69[c]	0.60[c]
SANITIZER (Chlorine)	0	0	1.23[d]	0.50[e]
TOTAL			2.96[e]	2.38[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

Table 39 - Log kill of attached Bacillus cereus from mixed culture exposed to 200 ppm acid anionic sanitizer in a simulated milk pipeline system (Log cells/cm^2; n = 2)

	MEAN ATTACHMENT		LOG KILL	
	RUBBER	TEFLON	RUBBER	TEFLON
INITIAL	3.70	3.78		
PRE-RINSE	2.89	2.87	0.81[a]	0.91[a]
CLEANER	1.49	2.27	1.39 [b]	0.59[c]
POST-RINSE	1.25	1.26	0.24 [c]	1.01[d]
SANITIZER (Acid Anionic)	0	0	1.25[d]	1.26[d]
TOTAL			3.70[e]	3.78[e]

* Means across each row with different letters are significantly different ($P < 0.05$)

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.

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