ATTACHMENT OF BACTERIA TO TEFLON AND BUNA-N-RUBBER GASKET MATERIALS

by

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

DOCTOR OF PHILOSOPHY

in

Food Science and Technology

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February, 1991
Blacksburg, Virginia

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(ABSTRACT)

Surface analysis of buna-N-rubber and teflon was performed. Scanning electron microscopy was used to analyze the topography of both materials and x-ray microanalysis identified the elemental chemical composition of the polymers. Teflon was primarily a smooth surface with random irregular spots, while buna-N-rubber had a very rough topography with "caverns" and crevices spread over the surface. The x-ray microanalysis showed that there are no impurities on the surface of teflon; however, calcium, silicone and sulfur were present on the surface of buna-N-rubber. Water contact angle measurements indicated that buna-N-rubber was a more hydrophobic surface than teflon.

Qualitative analysis of the attachment of Pseudomonas fragi ATCC 4973, Listeria monocytogenes Scott A and Bacillus cereus ATCC 11778 to buna-N-rubber and teflon was assessed by scanning electron microscopy. These bacteria readily attached to both surfaces. Pseudomonas fragi attached after 2 hours in the presence of this microoorganism and Bacillus cereus and Listeria monocytogenes attached at 12 and 24 hours, respectively.

Quantitative analysis of the attachment of Pseudomonas fragi to both surfaces as affected by various milk fat concentrations and temperature, and the availability of nutrients (different dilutions of skim milk, casein, casein and lactose,

and whey and lactose) was conducted. Attachment was assessed by impedance microbiology. Milk fat content did not play a significant role in the process of attachment of this organism to either type of surfaces; however, significantly greater numbers attached to buna-N-rubber than to teflon. Overall bacteria attached in higher numbers to both surfaces when grown at 21°C, compared to bacteria grown at 4°C. For buna-N-rubber, bacteria attached in significantly higher numbers when the concentration of nutrients was minimal, while for teflon, the results were, in most cases, opposite to these.

ACKNOWLEDGEMENTS

I would like to thank all the members of my committee, Dr. M.Pierson (Committee Chairman), Dr R. Bishop, Dr. R. Grayson, Dr. B. Eigel and Dr. J. Palmer., for their help at all times. Special thanks to Dr. M. Pierson for financial support and his time amidst his very busy schedule, and to Dr. R. Bishop for trusting to me his project, financial support and for the use of his portable computer. Thanks are also due to all the faculty, staff and fellow students in the Department of Food Science and Technology, for their help and friendship. Special thanks to Joe Boling for the statistical analysis of the results of my experiments, and to Kyle Thornham for technical assistance. Thanks to the Portuguese-American Foundation for their financial support during the most part of my studies, and to my friends for being there in the difficult times. Last, but not least, my deepest gratitude to my children Miguel, Tiago and Alan for their unconditional love and understanding, never complaining through the worst times of adversity. I dedicate this dissertation to them, and to all the Peoples around the world who may be suffering, in the hope that Universal Love and Compassion soon overcome human greed and intolerance, making this Planet a kinder Home for All.

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I. Introduction

Food contact surfaces can be an important source of contamination during food processing (Dunsmore and Bates, 1982; Lewis and Gilmour, 1987), and the final microbiological quality of food products may depend on the microbial population associated with those surfaces (Lewis and Gilmour, 1987). It is then imperative that surfaces in contact with those commodities are free as possible from microorganisms. In a milk processing plant and, in quantitative terms, stainless steel is the major food contact surface. Previous studies have shown that various bacterial contaminants in milk readily attach to milk contact surfaces (Herald and Zottola, 1987; Lewis and Gilmour, 1987; Zoltai et al., 1981). Gasket materials made from teflon and buna-N-rubber are widely used in pipeline connections in milk processing plants. The positioning and configuration of the gaskets make these materials difficult to clean and sanitize.

Bacillus cereus, Pseudomonas fragi and Listeria monocytogenes are environmental psychrotrophic contaminants, and have long been of concern in the dairy industry. Under normal production conditions, the predominant psychrotrophs found in raw and pasteurized milk are aerobic, motile, gram-negative rods of the genus Pseudomonas (Bigalke, 1984; Cousin, 1982; Mikolajcik, 1979). Their presence and growth in milk and dairy products leads to product spoilage and economic losses. Pseudomonas fragi is a common contaminant of raw milk and has been reported to attach to stainless steel surfaces (Zoltai et al., 1981; Scwach and Zottola, 1982).

Bacillus cereus is a motile gram-positive rod that produces endospores. Bacillus cereus has long been recognized as a human pathogen (Hauge, 1950, 1955), and common contaminant of milk and cream (Donovan, 1959; Billing and Cuthbert,

1958; Stone and Rowlands, 1952; Overcast and Atmaran, 1974). More recently, Ahmed et al. (1983) isolated this organism from samples of raw milk (9%), pasteurized milk (35%), Cheddar cheese (14%),and ice-cream (48%). Since this organism produces endospores, some concern has been voiced about the survival of spores in ultra-heat-treated milk (Franklin, 1970; Mostert et al., 1979) as well as their fate during the cheese making process (Mikolajcik et al., 1973).

Listeria monocytogenes is a motile gram-positive rod, which is a potential pathogen and is widely distributed in the environment (Cox et al., 1989). In recent years this organism has caused great concern in the dairy industry, due to food borne disease outbreaks caused by this microorganism where dairy products were the vehicle of infection (Becker et al., 1987; Braza, 1985; Fleming et al., 1985; James et al., 1985).

Listeria monocytogenes, Pseudomonas fragi and Bacillus cereus were chosen as representative psychrotrophic microflora of milk contaminants to determine if bacteria attach to gasket materials such as buna-N-rubber and teflon, making gaskets a possible source of contamination in dairy processing plants.

II. Literature Review

A. Introduction

Attachment of bacterial cells to solid surfaces is an ubiquitous phenomenon, and in nearly every eco-system studied, bacteria prefer to grow on surfaces rather than on the aqueous phase surrounding them. As a result, in microbial habitats of natural, medical and industrial importance, a greater number of bacteria grow attached to surfaces than in the fluids surrounding them (Costerton and Lappin-Scott, 1989).

The first report on the subject of bacterial attachemnt was done in 1913 by Sohngen, a Dutch microbiologist, on various experiments relating to microbial activities in soil, quoting earlier work done in 1908 on the physiology of attached bacteria. Heinrich (1933) and ZoBell (1943) studied the growth of marine bacteria and noted a strong tendency of these cells to grow on surfaces. They indicated that these solid-water interfaces would be expected to favor bacterial colonization because they concentrate nutrients. ZoBell (1943) also reported that bacterial counts were higher on the interior surface of a glass bottle than within water, and suggested that bacterial adhesion was a two-step phenomenon. He recognized an initial reversible phase, followed by an irreversible binding of microorganisms to surfaces.

Very little interest was expressed in the process of bacterial attachment to surfaces until the early 1970s, when researchers from various fields recognized that the association of microorganisms with surfaces and with each other was a widespread phenomenon (Marshall, 1976). At present, research on this area covers a variety of fields and major contributions have been made by microbiologists, engineers, physical chemists and physicists (Marshall, 1984).

Bacterial attachment to solid surfaces has been investigated in aquatic

environments (Fletcher and Floodgate, 1973; Johnson and Sieburth, 1976; Marshall et al., 1971 a), in terrestrial habitats (Balkwill and Casida, 1979; Dart et al., 1969; Marshall, 1976; Marshall et al., 1975), in bovine rumen (Akin, 1976; Cheng et al., 1976), in dental plaques (Gugenheim, 1970; Rutter, 1979), in infections of mucosal surfaces (Costerton et al., 1987; Ellwood and Tempest, 1972; Marrie and Costerton, 1985), indwelling medical devices (Costerton, 1984; Costerton et al., 1987; Gristina et al., 1985; Jacques et al., 1986; Marrie and Costerton, 1983 a,b,c), in food systems (Carson et al., 1987; Dunsmore and Bates, 1982; Dunsmore et al., 1981; Firstenberg-Eden, 1981; Firstenberg-Eden et al., 1978; Lilliard, 1985; McMeekin and Thomas, 1978; Notermans and Kapelmacher, 1974; Speers et al., 1984; Zoltai et al., 1981).

Before bacteria can attach to any kind of surface they have to be transported from the bulk of the liquid phase to the proximity of the solid-liquid interface.

B. Transport of Bacteria to Surfaces

When bacteria are dispersed in fluids, they will adhere to surfaces in conditions of turbulent flow, as well as in those of still fluids. The mechanism of bacterial transport to the surface is different in each case (Marshall, 1985). If the conditions are those of turbulent flow, the initial steps of bacterial adhesion to surfaces are influenced by the numbers of bacteria present in the fluid. Bryers and Characklis (1981), reported that under flow conditions the rate at which biofilm is formed is directly related to the concentration of biomass in the fluid phase; the rate of initial biofilm formation increased when the dispersed biomass increased. Characklis (1981a) found that in very dilute suspensions of bacteria, the rate-limiting phase during the process of initial deposition of bacteria onto a surface is probably the transport phase.

In conditions of turbulent flow, the viscous sublayer is recognized as a zone of relatively still fluids that exists close to the solid surface. The thickness of this layer is a function of the fluid shear rate. Characklis (1981b) showed that when the stress of shear is 6.5 to 7.9 N/cm^2, the viscous sublayer should be in the vicinity of 40 μm. When bacteria are suspended in the bulk of the liquid phase, they must cross this viscous sublayer if bacterial deposition onto the solid surface is to occur.

Various other mechanisms of bacterial transport to surfaces have been recognized (Marshall, 1985, 1976).

1. Cell surface hydrophobicity

Bacteria present a variable amount of relative cell surface hydrophobicity (Mudd and Mudd, 1924). More recently, these findings were confirmed by various research groups (Marshall and Cruickshank, 1973; Magnusson et al., 1977; Rosenberg et al., 1980; Dahlback et al., 1981). Marshall and Cruickshank (1973) suggested that hydrophobic bacteria may be excluded from the aqueous phase, thus leading to an attraction to any non-aqueous phase. According to the same authors, hydrophobicity could then be a way by which bacteria are attracted to solid surfaces.

Fattom and Shilo (1984) reported that bacterial cell surface hydrophobicity is a very important factor in the process of attachment of cyanobacteria to surfaces. Loosdrecht et al. (1987), also found that hydrophobic cells adhere better to sulfated polystyrene than hydrophilic cells. Cell surface hydrophobicity was measured as the contact angle of water on a bacterial layer. The value of contact angle was directly correlated to the number of cells attached to the surface. VanHaecke et al. (1990) studied the attachment of fifteen different isolates of Pseudomonas aeruginosa to stainless steel when related to cell surface hydrophobicity, and reported cell surface

hydrophobicity to be the major parameter influencing bacterial attachment during the first 30 minutes.

2. Sedimentation

Marshall (1973, 1976) regards bacteria as living colloid particles, presenting a density only marginally greater than that of water. As a result, in dilute electrolytes such as nutrient media, bacteria form stable suspensions partially due to electrostatic repulsion between similar charges present on the surfaces of bacteria. These stable suspensions only sediment when the system is destabilized such in floc formation.

Sedimentation is important only in cases of systems where the shear is low, and in the case of big particles, such as very large bacteria, or if bacteria aggregate and form flocs. Characklis (1981b) reported that under conditions of turbulent flow, sedimentation of bacteria is not likely to occur.

3. Brownian motion

Brownian motion is defined as a random movement of microscopic particles suspended in liquids or gases resulting from the impact of molecules of the fluid surrounding the particles (Webster's Dictionary, 1983.). The value of the particle displacement by Brownian motion ( ) can be calculated from the equation

$$\Delta = 2Dt$$

where D is the diffusion coefficient and t is the time in seconds. The diffusion coefficient D in inversely proportional to the radius of the particle.

$$D = RT/6\ aN$$

where: R-gas constant

T-absolute temperature

μ -viscosity of the medium

a-radius of particle

N-Avogadro's number

Larger bacteria do not show this kind of motion (Marshall, 1985), while small bacteria, with a radius smaller than 0.1μm, show an appreciable degree of Brownian displacement when observed under a microscope and in conditions of stillness. Since the effective radius of many bacteria is less than 1,0μm, Brownian motion provides individual cells with an important form of random motion, increasing that way the opportunities for bacterial deposition onto the surface by mere chance (Marshall, 1976). This motion is an important form of bacterial transport within the viscous sublayer (Characklis, 1981b); however, in conditions of turbulent flow is probably not important.

4. Chemotaxis

Chemotaxis is the ability of motile bacteria to move toward a chemical attractant, or away from a repellent (Pelczar et al., 1977). Motile bacteria display a positive chemotactic response to some sources of nutrients, Adler (1969) postulated the existence of cell-surface chemoreceptors that are involved in the detection of specific molecules, and has shown positive responses by Escherichia coli to galactose, glucose and L-aspartate. This positive chemotactic response allows bacteria to move towards a gradient of nutrients, such as the ones present in solid-liquid interfaces. It is possible that motile bacteria move in the direction of a surface, are positioned at the surface, and later held close to that surface by attractive forces (Marshall et al., 1971a). Motile bacteria may also show a negative chemotactic response from a solid-liquid interface when inhibitory substances such as antibiotics and hydrogen ions

accumulate there (Doetsh and Seymour, 1970; Seymour and Doetsch, 1973; Young and Mitchell, 1973b).

5. Fluid dynamic forces

In a system of turbulent flow, bacteria present in the fluid are dispersed from the central region by a process of eddy diffusion and, as a result, the concentration of bacteria in the bulk of the liquid phase is kept constant (Marshall, 1985). Bacteria are transported by eddy diffusion only until the viscous sublayer is reached. Once at the sublayer, different types of forces may be instrumental: if bacteria are travelling faster than the liquid in the region of the solid surface, a lift force will lead bacteria toward the solid surface (Characklis, 1981a). While in the viscous sublayer, bacteria may be subjected to a)frictional drag forces which will slow down the bacteria on their they approach to the solid surface, b) fluid drainage forces which are due to the resistance encountered by bacteria near the solid surface, and this resistance is attributed to the pressure in the draining fluid film, between the solid surface and the surface of the bacteria approaching it (Characklis, 1981a), and c) turbulent "downsweeps", spontaneous bursts of turbulence, also direct particles of bacterial size through the viscous sublayer (Lister, 1981) that provide an important fluid mechanical force to direct bacteria to the solid surface.

C. Bacterial Attachment, Aggregation and Biofilm Formation

Attachment of bacteria to solid surfaces and subsequent bacterial aggregation involve certain interactions between microorganisms and surfaces in an aqueous environment. These surfaces (substrata) may be varied: inorganic or organic (Noorlander and Heckman, 1980; Zoltai et al., 1981), animate or inanimate

(Noterman et al., 1974; ZoBell, 1943). The environment can be any type that supports microbial life (Akin, 1976; Costerton, 1984; Elwood and Tempest, 1972; Gugenheim, 1970; Marshall, 1976; Marshall et al., 1971; Firstenberg-Eden, 1978), and the microorganisms may be from one species only, or from various species (Costerton et al., 1985).

In natural habitats, bacteria will attach firmly to surfaces, and the immobilized bacteria grow, forming microcolonies and produce exopolymers which very often extend from the cells and form confluent slippery films called "slime layers" (Characklis, 1984; Costerton, 1981a; Gristina, 1989; Jones et al., 1969). Environmental debris will mix with the various layers of the slime and form biofilms, which may cover the surface evenly or may be "patchy" (Characklis, 1984; Costerton et al., 1985, 1981a; Fletcher, 1985; Jones et al. 1969; Shapiro, 1988). Biofilms function as a multicellular communicating milieu, and are a universal feature found in environments that support life (Costerton et al., 1985, 1981a; Fletcher, 1985; Jones et al., 1969; Shapiro, 1988). These biofilms incorporate ions and organic molecules from the environment and also hydrated anionic polysaccharides and glycoproteins produced by bacteria. Biofilms may be formed by a monolayer of cells or they may be 30-40 cm thick as in algal mats (Characklis, 1984). The simplest biofilms consist of microbial cells and their products. A biofilm may be formed by one species of bacteria only or by more than one species. The composition and properties of the biofilm depend on the bacteria present (Costerton et al ,1981a; Costerton and Lappin-Scott, 1989), leading to differences in confluent bacterial microcolonies. In this way, bacterial colonization transforms a homogeneous surface into a very heterogeneous surface.

Recently most of the attention given to biofilms has been focused on their

undesirable roles such as in the medical or dental areas where they are responsible for health problems (Beachey, 1980; Costerton, 1981a, b), and in the industrial area where they may cause fouling of equipment (Characklis, 1984). Biofilms can have a beneficial role such as in natural environments and engineered biological systems (Atkinson, 1984; Characklis, 1984).

1. Bacterial glycocalix

Costerton and co-workers (1981a, b), defined glycocalix as the structures of bacterial origin containing polysaccharides, which lie outside of the peptidoglycan in gram-positive cells and outside of the outer membrane of gram-negative cells. These structures evaded scientists for some time, as they were not properly visualized in earlier electron microscopy work, the reason being that polysaccharides forming the glycocalix do not effectively attract heavy metal stains. The use of ruthenium red, a polyanion-specific stain (Luft, 1971), and alsian blue (Fletcher and Floodgate, 1973) enabled the visualization of bacterial glycocalixes. These appear as regions on the bacterial cell surface where the density of electrons is very high, due to the process of dehydration used for sample preparation in electron microscopy. This process almost completely destroys the polysaccharide network which in natural conditions is 95-99% water (Characklis, 1984; Sutherland, 1977).

More recently, different techniques involving the use of specific antibodies (Chan et al., 1982a; Mackie et al., 1979), and lectins (Birdsell et al., 1975), have allowed for a better description of the surfaces of bacterial cells. Dogget et al. (1964) reported a change from the wild nontypeable strains to the typeable, standard laboratory cultures, once bacteria were isolated from natural habitats and then grown in laboratory conditions. When bacteria are grown in vitro in optimal

conditions, the surfaces of bacterial cells are very different from those of bacteria grown under some kind of selective pressure. Govan (1975), found that the use of surfactants in media promotes glycocalix formation, and a similar response from bacterial cells was reported when antibiotics alone or in combination with surfactants were used in the growth medium (Govan and Fyfe, 1978). Costerton (1985), states that bacterial glycocalix formation is an universal phenomenon found in natural and pathogenic eco-systems, and these glycocalixes will mediate microcolony formation as well as adhesion. However, Paul and Jeffrey (1985), in their study with Vibrio proteolytica reported that no slime production was observed, suggesting that these organisms may represent the primary type of fouling in aquatic environments and slime production may occur later.

2. Mechanisms of bacterial attachment

Two main approaches have been taken in trying to explain the very complex phenomenon of bacterial attachment to surfaces: the surface energy approach (Baier, 1980; Carson and Allsopp, 1980; Dexter et al., 1975; Dexter, 1979; Fletcher and Loeb, 1976; Pringle and Fletcher, 1983), and the DLVO (Derjaquin and Landau, 1941; Verwey and Overbreek, 1948) theory of colloid stability (Hamada, 1977; Marshall et al. 1971a; Pethica, 1980; Rutter and Vincent, 1980; van Houte and Upeslacis, 1976).

a. Surface energy approach

The wettability of a substratum has been defined in terms of the surface free energy and in terms of hydrophobicity.

The surface free energy can be estimated by:

. measuring the contact angle (Neuman et al., 1979, 1974),

. finding the sum of two components of the surface free energy, the

dispersion and polar components (Wu, 1980),

. calculating the critical surface tension (Zisman, 1964).

Hydrophobicity may be characterized in terms of:

. contact angle of water (Fletcher and Loeb, 1979),

. as the adhesion work between the surface and water (Pringle and Fletcher, 1983).

Baier (1973, 1980) suggested that in aquatic environments, bacterial adhesion should be minimal when substrata are relatively hydrophobic, having surface tensions of 20 to 30mN/m. He called this range "minimally bioadhesive". Results obtained by Dexter (1979) and Dexter et al. (1975) showed minimal bacterial adhesion to hydrophobic substrata, and maximal bacterial adhesion to high energy (hydrophilic) surfaces, thus confirming Baier's prediction. Others (Carson and Allsopp, 1980; Loeb, 1977) have reported that on surfaces immersed in sea water, colonization of hydrophobic surfaces was faster than that observed on hydrophilic surfaces. Fletcher and Loeb (1979, 1976), found that large numbers of a marine pseudomonad attached to hydrophobic surfaces with little or no surface charge, such as teflon and polystyrene. They reported that moderate numbers of bacteria attached to hydrophobic metals with a positive charge (platinum) or neutral (germanium) surface charge and very few bacteria attached to hydrophilic negatively charged surfaces such as glass and mica. The increase in attachment was directly correlated to the increase of water contact angle value (Fletcher and Loeb, 1979). Pringle and Fletcher (1983) studied freshwater bacteria in laboratory conditions and found that the majority of microorganisms attached preferentially to hydrophobic surfaces; however, no pattern off attachment was reported. Each bacterium attached in maximum numbers to a surface that was specific for that

given organism. In another report, Pringle and Fletcher (1986) reported that hydration of a surface may reduce bacterial attachment; however, other factors such as chemical composition of the substratum and stabilization of suspended cells can affect adhesion interaction, thus influencing the final numbers of attached cells. These authors also suggested that hydrogels bind water layers more efficiently than clean substrata. The adsorbed water molecules could reduce the free energy of the surface-liquid interface, change the ionic environment and physically prevent bacteria to closely approach the surface.

To complicate matters, Paul and Jeffrey (1985) found that Vibrio proteolytica may have two different adhesion mechanisms, one for hydrophilic surfaces and another one for hydrophobic surfaces.

b. DLVO theory of colloid stability

The DLVO theory of colloid stability was described independently by Derjaquin and Landau (1941) and by Verwey and Overbreek (1948), and was latter revised and adapted to explain bacterial attachment to surfaces (Rutter and Vincent, 1980; Pethica, 1980). According to this theory the interaction of any two particles is a function of two components which are additive: a repulsive component, when the particles have the same charge sign, this component is responsible for the stability against aggregation; and an attractive component which is due to van der Waals forces, and is the principal cause of aggregation.

The repulsive component is attributed to the overlap of the electrical double layers, and these depend on the groups of charge present on the two particles. Attraction is only possible at two well defined distances of particle separation, the primary minimum where attraction forces are strong and this distance is a very small distance between particles, and at the secondary minimum where attraction

forces are weaker, this distance between particles is relatively large. The particle radii, surface potentials electrolyte concentration and Hamaker constant (dependent on the composition of particles and separating medium), all are instrumental in determining the total interaction between particles at any given distance. The repulsion barrier depends on the electrolyte concentration, decreasing when electrolyte concentration increases, and eventually disappearing at high electrolyte concentrations.

The concept of "double layer" as proposed by Stern defines a certain net charge distribution on the solid surface, resulting from ionization of functional groups and from preferential adsorption properties. In a certain finite region, electrical neutrality is attained through the concentration of counterions (ions of opposite charge) from the liquid, around the specifically charged surface ions. This leads to the fact that counterions of the liquid are more concentrated around the surface than ions of similar charge. The Stern layer is formed by those counterions that are held close to the surface, and is normally one molecule thick.

3. Types of bacterial attachment

In general terms two main types of bacterial attachment to surfaces have been defined: reversible and irreversible attachment, (Marshall et al., 1971a).

a. Reversible attachment

This type of attachment is a reversible, non-specific adhesion, due to general long-range forces which hold bacteria close to the surface (Marshall et al., 1971a). These long range forces depend on the physical characteristics of the bacterium, the fluid interface and the substratum. During this phase, bacteria still show Brownian motion and the shear force of a jet of water is enough to displace them from a

surface (Marshall et al., 1971a). ZoBell (1943) was the first to identify reversible attachment and this phenomenon has since then been studied by other groups (Hamada, 1977; Jones et al., 1980, 1981; Marshall et al., 1971a; van Houte and Upeslacis, 1976).

At pH values found in the majority of natural habitats, most bacteria present a negative surface charge (Marshall et al., 1971a), and most substrata also display a negative surface charge. This fact may appear to pose a problem; however, the DLVO theory of colloid stability (Derjaquim and Landau, 1941; Pethica, 1980; Rutter and Vincent, 1980; Verwey and Overbreek, 1948) offers some basis for explanation of this phenomenon. The like charges presented by the bacterium and the surface, cause them to repel each other; at the secondary minimum (approximately 10nm), van der Waals forces hold the bacteria close to the surface (Dankert et al., 1986; Fletcher, 1985; Gristina, 1989; Jones and Isaacson, 1984; Marshall et al., 1971a). Marshall et al. (1971a) reported that repulsion of Achromobacter R8 to glass surfaces increased as the electrolyte concentration decreased. The same group of workers suggested that at high electrolyte concentrations, bacteria should show a reversible attraction at the secondary minimum. Repulsion of bacteria still occurs until the primary minimum (distance of less than 2 to 3nm between surfaces) is reached. Here attraction forces occur (Dankert et al., 1986; Fletcher, 1985;Jones and Isaacson 1984; Pashley et al., 1985). At distances of 15nm hydrophobic forces are active. At distances of 8-10nm, these forces are 10-100 times greater than van der Waals forces (Dankert et al., 1986; Jones and Isaacson, 1984). These attractive hydrophobic interactions overcome residual like-charges forces and lead the bacteria to the primary minimum. Gristina (1989) suggested that at distances of 1nm or less from the surface, short-range chemical

interactions such as ionic, covalent, and hydrogen bonding are active between ionic adsorbates, glycoproteins, surface atoms and extracellular structures.

Christensen et al. (1985) reported that in the case of bacteria-to-tissue cell pathogenesis, simultaneously with initial attachment and, if present, specific fimbrial adhesins and surface receptors such as adsorbed glycoproteins may interact. Stenstron (1985) suggested that fimbriae may also react nonspecifically with the substratum, either by charge or hydrophobic interaction.

b. Irreversible attachment

Zo Bell (1943) recognized that once bacteria are attracted to a surface, they become strongly attached to that surface due to the biosynthesis of extra-cellular adhesive materials. Hirsh and Pankratz (1970), found bacteria to be firmly attached to electron microscope grids through amorphous, granular, holdfast bacterial structures.

It was Marshall and co-workers who in 1971 (a) defined irreversible attachment as a time dependent firm adhesion, where bacteria did not show Brownian motion any longer, and could not be removed by washing. They also suggested that polymer bridging was responsible for the strong hold of bacteria onto the surface, these polymers being regarded as a bacterial response to nutrients accumulated on the surface. Polymer bridging between surfaces and bacteria was later confirmed by Marshall and Cruickshank (1973) and by Fletcher and Floodgate (1973), who also reported the presence of acidic polysaccharide material. These latter authors described two types of polymers produced by a pseudomonad attached to surfaces: on the outer surface of the cell wall a compact layer was present, while another layer of loose fibrous polysaccharides was adjacent to the first one. Fletcher and Floodgate (1973) suggested that the compact layer was involved in the initial

bacterial attachment and the second layer may have been produced after the initial attachment took place. Costerton et al. (1981a, b) also reported that after initial adhesion of bacteria to surfaces, some additional polymers are synthesized. Tadros (1980) reported that when extracellular polymers bind to surfaces, short-range forces are very important, and divided these forces into various groups:

. chemical bonds such as hydrogen, covalent and electrostatic bonds,

. hydrophobic bonding,

. dipole interactions such ion-dipole and dipole-dipole interactions.

More recent studies suggest that the time dependent biosynthetic chemical processes of bacteria which lead to irreversible attachment depend partially on specific protein adhesins, adhesin-receptor interactions, lectins and also on direct chemical interaction with the surface (Fletcher, 1985; Gibbons and van Houte, 1980; Jones and Isaacson, 1984).

Gristina (1989), reported that some biomaterial surfaces may have a catalytic affect on irreversible attachment of bacteria due to the fact that energy for molecular interactions may be obtained from thermal vibrations of the underlying lattice. Molecular interactions are far more frequent on planar surfaces than in three-dimensional systems.

Some researchers have defined ways to measure the strength of bacterial attachment to surfaces. Tadros (1980) and Zvyagintsev et al. (1977) reported a method based on the application of a centrifugal force which would balance the attachment force of the bacteria. Marshall et al. (1971a) found that mechanical removal of the bacteria from a surface left some materials attached to the surface which the authors called "footprints". Marshall (1985), however, cautions that measurements of bacterial adhesive strengths may not be precise due to fact that

they may not evaluate the real adhesive forces, and also for the same bacterium adhesive strengths may vary for different surfaces.

4. Bacterial extracellular polymers

Exopolysaccharides of slime-producing bacteria are an amorphous material composed of various polymers of high and low molecular weight that are associated by ionic interactions (Gristina, 1989). These exopolysaccharides are formed from amino-sugars, uronic acid, polyols such as ribitol and glycerol, and neutral monosacharides such as D-glucose, D-mannose, D-galactose, L-ramnose and L-fucose (Sutherland, 1980). Ichiman and Yoshida (1981) and Peters et al. (1987), reported on the monomeric carbohydrate units and some amino-acids of Staphylococcus epidermidis slime: Glucose, galactose, glycerol, hexosamine, phosphorus, glycine, alanine, and phenylalanine were found to be the major components. Slusher et al. (1987) while studying the composition of exopolysaccharides of Staphylococcus epidermidis, strain SE-360 found mannose in addition to all the other compounds referred above. Proteins may also be a part of the attachment polymers produced by bacteria (Danielsson et al., 1977). These authors were able to remove a marine pseudomonad from surfaces by treating the surface with proteolytic enzymes. However, Marshall (1973) and Fletcher (1980b) reported that they were unable to remove a marine bacteria from glass surfaces using proteolytic enzymes. Fletcher and Marshall (1982) used pronase and successfully removed Pseudomonas NCMB 2021 form polystyrene surfaces but not from glass surfaces, indicating that polymer bridging in this organism may involve different mechanisms of biosynthesis for different surfaces. Evidence of two different mechanisms for attachment to hydrophobic and to hydrophilic surfaces was also reported by Paul and Jeffrey

(1985).

Extracellular polymers, "slime", which enclose biofilms play an important role in the persistence of infections (Costerton, 1985; Gristina, 1989). Most have polyanionic matrices (Sutherland, 1977), which act as an ion exchange resin, allowing for greater nutrition and decreasing susceptibility to phagocytosis. In this way, surface-adherent bacteria live in a protected environment that is formed by the surface itself, the overlying biofilm and all the trapped ions, metabolites and glycoproteins. This favorable niche changes the bacterial sensitivity to antibiotics when compared to bacteria living in suspension. Gristina (1989) reported that once Staphylococcus epidermidis and Staphylococcus aureus are attached to surfaces, the minimum bactericidal concentration for various antibiotics increases 2-250 fold, depending on the type of surface to which the bacteria were attached.

5. Substratum conditioning and degradation

The presence of macromolecules on the aqueous phase has various affects on the process of bacterial attachment; it may inhibit, enhance or not affect it (Fletcher, 1976; Meadows, 1971; Orstavik, 1977). Maroudas (1975) proposed that when macromolecules adsorb to a surface, the surface free energy will be altered and possibly steric exclusion effects are also created. Fletcher and Marshall (1982) reported a bubble contact angle method to evaluate the changes observed in the surface free energy values, when the surface was exposed to an aqueous phase where macromolecules were incorporated. Bubble contact angles at the surfaces exposed to the solution containing macromolecules varied with the concentration and composition of the solution. The role of dissolved and substratum-adsorbed proteins (bovine glycoprotein, bovine serum albumin, fatty acid-free bovine serum albumin)

on bacterial attachment to surfaces of Petri dishes and of tissue culture dishes was varied: in the case of tissue culture dishes bacterial attachment was correlated to changes in bubble contact angle values, unless proteins were adsorbed to the tissue culture dishes prior to bacterial attachment. In the case of Petri dishes, results were not consistent with those described, suggesting two different mechanisms of attachment to the two substrata. In another study, Pringle and Fletcher (1986) found that adsorption of macromolecules to the surface may reduce bacterial attachment; however, these authors propose that other factors may also be envolved.

Functionally, surfaces are interfaces that concentrate charged particles, molecules and nutrients from organic and mineral sources, or the surfaces themselves may be metabolized (Baier, 1979; Gristina, 1989; ZoBell, 1943). Microzones are specific environmental and metabolic microclimates that exist within a biofilm, colonizing a certain surface (Paerl, 1985). In these microzones optimal conditions exist and antagonistic environmental factors are excluded.

Ions such as Fe^{3+}, Mg^{2+}, Ca^{2+}, and Co^{3+} may be available in varying amounts after corrosion of metallic surfaces. Stable alloys such as stainless steel can be corroded to a certain degree due to wear or chemical interactions with a non-favorable biological environment. Gristina et al. (1987) found traces of iron, aluminum and other substances due to manufacturing processes or to contamination. Trace ions such as Ca^{2+} and Mg^{2+} may also be present and possibly by reacting with acidic groups, may be instrumental in stabilizing complex bacterial exopolysaccharides in a gel state, reinforcing cell-to-cell and cell-to-surface aggregation and attachment, thus increasing resistance to exogenous hostilities (Dankert, 1986; Fletcher, 1985).

According to Gristina (1989), it is possible that unstable polymers could be

directly metabolized or give off monomers, antioxidants and stabilizers. Ludwicka et al. (1983) reported that ester bonds are present in some synthetic polymers such as polyester urethane and methylmethacrylate, and those ester bonds may be hydrolyzed by staphylococci. It is also possible that Ca^{2+} may be deposited or crystallize on surfaces of polymers, thus leading to an alteration of mechanical properties and disintegration of the surface (Gristina, 1989).

Costerton and Lapin-Scott (1989), reported that sulfate-reducing bacteria are the main cause of metal corrosion, suggesting that these bacteria develop biofilm-generated corrosion cells by establishing anodes and cathodes on the metal surface which allow corrosion currents to take place. These cathodes and anodes are due to different metal ion concentrations originated by microbial growth products exuded into the biofilm. Once the cathodes and anodes are established, measurable corrosion potentials will develop, thus leading to metal loss at the anode. Bacterial corrosion, a biofilm generated process, may perforate steel plates of 5/8 inch thickness in less than 6 months (Costerton and Lapin-Scott, 1989).

6. Influence of bacterial growth conditions on attachment

Fletcher, (1977), found that a marine pseudomonad attached better to surfaces when the bacterial cells were in log phase, and noted a progressive reduction of numbers of bacteria attached, once stationary and death phases were reached. Zvyaginstsev et al. (1971, 1977) reported similar findings with the decrease in attachment strength directly correlated to an increase of culture age. Stone and Zottola (1985) observed an increase in attachment of Pseudomonas fragi during log phase, both in stationary and dynamic conditions of bacterial growth.

Bacterial attachment may also be affected by the nutrients available.

Marshall (1971a) reported the attachment of a marine pseudomonad to be dependent on the concentration of the glucose, used as a carbon source in the study. Attachment was favored at glucose concentrations of 7 mg/l, while at concentrations of 14 and 21 mg/l it was reduced and completely inhibited at glucose levels of 30 and 10 mg/l, respectively. The authors also observed that at the lowest concentration of glucose studied, 7mg/l, polymeric fibrils attaching bacteria to the substratum were more prevalent than at higher concentrations of glucose, where polymer production was variable.

D. Bacterial Attachment in Foods

During the early 1970's, food microbiologists began studying bacterial attachment to foods and food contact surfaces; however, compared to other areas such as medical and dental, only a relatively small number of studies have been performed.

Generally speaking, bacterial attachment to any kind of surface is a very complex phenomenon and the mechanism of bacterial attachment to foods is no exception. Notermans and Kampelmacher (1974) suggested that during processing, bacteria attach to skin of broiler chickens. They studied various parameters of the attachment medium: numbers of bacteria, pH and temperature. Their results indicated that: a)the rate of attachment is directly correlated to the number of bacteria present in the attachment medium, b)the effect of pH on the rate of attachment varies with the microbial species and c)the attachment rate is maximal at 21° C . These authors also found that flagellated bacteria consistently attach to poultry skin while non-flagellated bacteria rarely attached.

Since then a number of research groups have performed various studies and

results have not been consistent. Thomas and McMeekin (1984, 1981a, 1980) found bacteria not to attach to the skin of chickens, they proposed that spoilage microorganisms grow in a liquid film covering the skin surface and in the feather follicle shafts, suggesting that skin microtopography and the presence of the liquid film were the major factors controlling contamination during processing. These authors reported changes in the microtopography of the poultry skin to be caused by water uptake during processing (immersion). Those changes were dependent on the temperature and time of immersion. In breast skin, water absorption caused "capillary-sized channels and spaces" to open in the surface layers. Due to those changes, more water was retained as a surface film when compared to tissues immersed for only short periods of time. They proposed that numbers on contaminating bacteria might be expected to increase due to the increase of the liquid retained in the surface film (Thomas and MCMeekin, 1984). In another study, Thomas and McMeekin (1981a) found Salmonella sp. to attach to the collagen fibers of chicken immersed for extended periods of time in water. The authors also reported that the presence of sodium chloride in the suspension medium prevented bacterial attachment.

However, McMeekin and Thomas (1978) reported that numbers of bacteria "retained" in the skin was directly proportional to the numbers of bacteria present in the testing suspension. They proposed that bacterial retention did not increase with time of immersion. Butler et al. (1979), Chung et al. (1989) and Lillard (1985), in studies on beef and lamb, beef and chicken meat, respectively, reported that bacterial attachment to meat surfaces increased when the concentration of organisms in the attachment suspension was also increased. Lillard (1986b) also found that as the time of immersion of poultry skin increased, the percentage of bacteria in the liquid film decreased, while the percentage of bacteria on the skin increased. This

suggested a transfer of water and bacteria from the surface film to the skin in conditions of prolonged water immersion.

Firstenberg-Eden et al. (1979) and Notermans et al. (1979) in their studies on bacterial attachment to the surface of cow teats, reported the process to be a time dependent two-step phenomenon involving bacterial flagella, capsule formation, and extra-polymeric adhesives.

Bacteria attach faster to some meats than to others (Firstenberg-Eden et al., 1979; Notermans et al. 1979). Escherichia coli K12 attached best to chicken muscle with fascia than to other meats. There is a linear relationship between numbers of bacteria attached to surfaces versus time of exposure of meat to attaching suspensions, over a period of 60 minutes. In the case of cut chicken muscle and cut beef muscle, the increase of numbers of bacteria attached to those surface stop after approximately 20 and 30 minutes respectively.

Buttler et al. (1979) reported that after the first minutes of meat (beef and lamb) immersion in the adhering suspension, there is very little additional bacterial attachment. With Pseudomonas putrefaciens, Erwinia herbicola and Escherichia coli there is only a very small increase on the numbers of bacteria attached after the first minute. Chung et al. (1989) studied attachment of bacteria to lean meat and to fat tissue, and reported that all bacteria studied attached instantly to meat surfaces once meat samples were exposed to the bacterial suspension. When the incubation time was extended, a small increase in the numbers of cells attached to meat was observed for Listeria monocytogenes, but not for the other bacteria studied. No difference was found between the attachment of the different bacteria to lean or fat tissue.

The role of bacterial flagella in the attachment process has been controver-

sial. Buttler et al. (1979), Farber and Idziak (1984), and Notermans and Kampelmacher (1974), found flagellated bacteria to attach better to meat and poultry surfaces than non-flagellated bacteria. On the other hand, McMeekin and Thomas (1978) were unable to confirm these results and Lillard (1985) suggested that non-flagellated bacteria attach as well as flagellated bacteria to poultry skin. In another study Lillard (1986b) concluded bacterial attachment to poultry skin to be a very complex phenomenon which may involve other mechanisms besides fimbriae, flagella or water uptake.

The study of the effect of temperature and pH on the process of bacterial attachment has also been studied. Notermans and Kampelmacher (1974) reported attachment to be optimal at 21° C and pH 8.0, while Butler et al. (1979) found pH and temperature to have very little effect on the extent of bacterial attachment.

E. Bacterial Attachment to Food Contact Surfaces

During processing, storage and transport, foods are in contact with various surfaces and these form an important source of microbial contamination (Dunsmore and Bates, 1982; Lewis and Gilmour, 1987). Once attached to food contact surfaces, bacteria may survive the cleaning process, remain in the system and contaminate a new batch of product (Dunsmore et al., 1981; Notermans and Kampelmacher, 1974; Thomas and McMeekin, 1981a, 1980).

Noorlander and Heckman (1980) studied inner surfaces of new and old rubber and Silastic (silicone rubber) teat cup inflations and identified cracks and depressions in new rubber inflations which increased in width with use. No cracks were visible in the silastic inflations. Bacteria were present in the cracks of used rubber inflations, leading to believe that teat cup inflations can become a reservoir

for bacteria and these bacteria could be spread from cow to cow in a dairy herd via the milking machine. Zoltai et al. (1981) reported that all bacteria strains studied attached to glass and stainless steel surfaces. It was noted that Pseudomonas fragi showed fibrous material extending from the bacterial cell to the surface, which became more pronounced with contact time. In the case of all the gram negative bacteria used in this study, Zoltai et al. (1981) reported a lack of fibril production, suggesting a primary type of attachment. Dunsmore and Bates (1982) showed attachment of both gram-positive and gram-negative organisms to glass slides, and reported the rate of attachment to be dependent on the organism studied, with Escherichia coli attaching irreversibly almost immediately after the slide was exposed to the attaching suspension.

Schwach and Zottola (1982) and Carson et al. (1987) showed transfer of bacteria attached, respectively, to beef and raw poultry skin to stainless steel surfaces. Schwach an Zottola (1982) using scanning electron microscopy observed transfer of microorganisms from beef to stainless steel surface, and reported the formation of attachment fibrils after 4 hours of contact between the microbial cells and stainless steel. These attachment fibrils increased in size and in number after 24 hours. They suggested that bacterial attachment to stainless steel is a two step process. Using Salmonella labelled with ^{32}P , Carson et al. (1987) studied the transfer of that bacteria from raw poultry skin to stainless steel.

Speers et al. (1984) have shown bacterial attachment to dairy equipment surfaces such as rubber, glass and stainless steel, and suggested that topography of surfaces may be instrumental in the process of bacterial attachment. Schwach and Zottola (1984) found that when Pseudomonas fragi attaches to stainless steel chips, rinsing the contaminated surfaces with water or with sodium hypochlorite before

sanitizing, is not an effective method to remove attached microorganisms. The growth conditions of Pseudomonas fragi do not seem to play a role in the process of bacterial attachment to stainless steel (Stone and Zottolla, 1985a). Bacteria grown at 25°C and at 4°C both in stationary and dynamic environments readily attached to the surface, with attachment fibrils present in all cases. In another study, Stone and Zottola (198b) found that with a clean in place (CIP) system some cells of Pseudomonas fragi remain in the system; however, the remaining cells do not form attachment fibrils to the stainless steel. When the conditions are suboptimal, cells form attachment fibrils after growing in milk at 21°C for 24 hours and at 4°C for 96 hours.

Lewis and Gilmour (1987) reported that milk microflora attaches to internal surfaces of rubber and stainless steel. They did not find a significant difference between the numbers of bacteria attached to both surfaces. According to Herald and Zottola (1988), temperature and pH are important in the attachment of Yersinia enterocolitica to stainless steel, with higher numbers of bacteria attached at 21°C and pH 8,0. Attachment was also observed at 35°C and 10°C when the pH was 9.5. At pH 6.0, some cells attached when the temperature was 21°C but only occasionally when the temperature was 10°C or 35°C. The same authors also reported that dynamic growth conditions reduced attachment of Yersinia enterocolitica to stainless steel surface at all pH values studied.

These latter findings are not in agreement with those reported by Stone and Zottola (1985) for Pseudomonas fragi. Herald and Zotolla (1988) found that Listeria monocytogenes Scott A attached to stainless steel at 10°C, 21°C and 35°C; however, cells with fibrils were observed only at 21°C. The pH of the medium was important and when cells were grown at pH 8 the attachment matrix was more evident at 21°C than at 35°C. When grown in a dynamic environment, the attachment of Listeria

monocytogenes to stainless steel was reduced. A direct relationship between numbers of attached cells and incubation time was also reported.

Frank and Koffi (1990), studied the attachment of Listeria monocytogenes to glass slides and the heat and antimicrobial resistance of the attached cells. They found attached cells to be more heat resistance than cells in suspension. An increased resistance to various antimicrobials was also noted for attached cells when compared to cells in suspension. Mafu et al. (1990), reported that Listeria monocytogenes Scott A attached to stainless steel, glass, polypropylene and rubber surfaces. The attachment of this bacterium to those surfaces does not increase consistently with time; however, there is attachment at ambient and cold storage temperatures.

III. Objectives

A. Study the surface properties of buna-N-rubber and teflon gasket materials by the use of scanning electron microscopy, X-ray energy dispersive analysis and water contact angle measurements.

B. Conduct a qualitative analysis by scanning electron microscopy of the attachment of Pseudomonas fragi ATCC 4973, Listeria monocytogenes Scott A, and Bacillus cereus ATCC 11778 to buna-N-rubber and teflon.

C. Conduct a quantitative analysis of the attachment of Pseudomonas fragi to buna-N-rubber and teflon as affected by milk fat content and assessed by impedance microbiology.

D. Conduct a quantitative analysis of the attachment of Pseudomonas fragi as affected by content of nutrients (different dilutions of, respectively, skim milk, casein, casein with lactose, and whey with lactose), and assessed by impedance microbiology.

IV. Materials and Methods

A. Surface Analysis of Teflon and Buna-N-rubber

1. Scanning electron micoscopy and X-ray analysis

Scanning electron microscopy (SEM) was used to study the topography (Johari and Samudra, 1974). Cylindrical pieces of teflon (Du Pont, Wilmington, Delaware) and buna-N-rubber (M.G. Newell, Greensboro, N.C.) of 3.8 mm diameter and 2.0 mm and 4.0 mm height, respectively, cut with a bore-holer, were mounted on aluminum stubs, gold coated with a layer of 15 nm thick in a Hummer X sputter coater (Anatech Lta.), and viewed on a Phillips 505 scanning electron microscope. To determine the elemental composition of the bulk of the two gasket materials X-ray micro-analysis was performed (Woldseth, 1973): the same size pieces of both gasket materials as described above were mounted on aluminum stubs, carbon coated on a Kinney Vacuum KSE-2A-M Evaporator and analyzed with a Phillips 505 scanning electron microscope equipped with an Edax model P505-149-10, X-ray detector.

2. Contact angle measurements

The value of the water contact angle for both surfaces was obtained by direct measurement of the angles of water sessile drops. Teflon and buna-N-rubber surfaces were placed on the microscope stage of a Rame-Hart contact angle goniometer , model 100-00, and a micro-seringe (Rheodyne 705-SNR, Hamilton Company, Reno, Nevada) was used to deposit 10 1 drops of deionized water onto the respective surface. Measurements were obtained from three different sessile drops of water read on both sides. All measurements were performed within 5 seconds of depositing the liquid drop onto the surface.

B. Qualitative Analysis of Bacterial Attachment to Teflon and to Buna-N-rubber

Pseudomonas fragi ATCC 4973, Listeria monocytogenes Scott A, and Bacillus cereus ATCC 11778 were used in this part of the study, as representative psychrotrophic microflora.

Teflon and buna-N-rubber gaskets were cut into cylindrical pieces of 3.8mm diameter and 2.0mm and 4.0mm height, respectively. The cylindrical pieces were wrapped in aluminum foil and sterilized by autoclaving for 15 minutes at 121° C. The sterile gasket materials wre placed in 2% fat milk (commercial brand) that had been diluted 1:4 with distilled water and steamed for 10 minutes at 100°C. This dilution was done to prevent milk coagulation during incubation.

Erlenmeyer flasks (500 ml) containing 6 pieces of each gasket material and 250 ml of the diluted milk were separately inoculated with the following bacteria: Pseudomonas fragi, $1x10^7$ cells/ml grown overnight in Nutrient Broth (NB) at 21° C; Listeria monocytogenes $1x10^6$ cells/ml grown overnight in Trypticase Soy Broth suplemmented with 0.06% yeast extract at 37° C, and Bacillus cereus, $1x10^5$ cells/ml grown in NB at 31° C. The number of bacteria present in the three inocula was determined by optical density, using a standard curve relating numbers of bacteria (obtained by Standard Plate Count) with optical densities. The inoculated flasks were incubated at 21°C in a controlled environment shaker incubator, Model G-26 (New Brunswick Scientific Co. Inc.,Edison,N.J.), at 130 r.p.m.. This temperature was chosen due to the fact that it is the most common room temperature used in the dairy processing industry.

For Pseudomonas fragi samples of gasket material were taken after 2, 4, 6, 24, 48,and 72 hours of bacterial exposure. Autoclaved pieces of gasket material were placed in uninoculated milk, and samples were taken at the same time intervals. In

the case of Bacillus cereus and Listeria monocytogenes samples were taken at 2, 8, 12, 24, 48, and 72 hours of bacterial exposure.

All samples were prepared for scanning electron microscopy by vortex washing three times in a glass tube with 1 ml of 0.1 M cacodylate buffer at pH 7.3 (Hayat, 1972), to remove reversibly attached bacteria from the surfaces. Samples were then divided into two groups: one group was frozen at -40°C and dehydrated by freeze-drying on a Virtis freeze-drier model 10-145MR-BA and the other group was fixed in 2% glutaraldehyde, paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.3 (Karnovsky,1965), dehydrated in a alcohol series and then critical point dried in a critical point dryer (Ladd, Research Industries). Acetone was the intermediate fluid and carbon dioxide the transitional fluid. After drying, both groups of samples were coated with a 15 nm layer of gold on a sputter coater (Hummer X, Anatech, Lta.), and viewed on a 505 Phillips scanning electron microscope, at an accelerating voltage of 20 Kv.

In the case of Listeria monocytogenes only one group of samples was taken and treated for critical point drying, procedures as above.

C. Quantitative Analysis of Bacterial Attachment to Teflon and to Buna-N-rubber Surfaces as Affected by Milk Fat Content

Pseudomonas fragi ATCC 4973 was used in this part of the study. From the three microorganisms used previously, this bacterium is the one that grows better at the temperatures chosen for this study (21 and 4° C). These temperatures were chosen due to the fact that they represent the most commonly used temperatures in the dairy processing industry for , respectively, processing and storage. Commercial milk of different fat content was used: skim, 2%, 3.3% and 16% (half and half). The

fat content of this milk was confirmed by the Babcock method (Horwitz, 1980; Marth, 1978; McKinley, 1975; Newlander, 1977; Richardson, 1985). For each milk fat content the quantitative analysis of bacterial attachment was performed in triplicate, and in each experiment three replicas of each sample were used. Milk and half and half were steamed for 10 minutes at 100°C. Erlenmeyer flasks (250 ml) containing 9 sterile pieces of each gasket material and 100 ml of milk were inoculated with $1x10^7$ cells/ml of Pseudomonas fragi (prepared as described previously) and cultures were incubated at 21°C and at 4°C in a controlled environment shaker incubator at 130 r.p.m.. To prepare the inoculum Pseudomonas fragi was grown overnight at 21° C in NB. Samples of gasket pieces were collected after 12 hours of exposure and were processed as follows:

a. Three pieces each of teflon and buna-N-rubber were gently rinsed with standard methods dairy diluent (Richardson, 1985), and for each piece after rinsed, the number of bacteria attached to the surface was determined. The number of bacteria on the samples prepared by this procedure represent the total number of bacteria attached to the surface of the respective piece of gasket (reversibly and irreversibly attached).

b. Three pieces of each gasket material were gently rinsed with standard standar methods dairy diluent and then vortex washed three times in a sterile glass tube containing 1 ml of standard dairy products diluent during 30 seconds each time. The number of microorganisms in each of the three washings and on the pieces of gasket were determined individually. The number of bacteria present in each of the three washings represent the numbers of bacteria reversibly attached to the surface. The number of bacteria on each of the gasket pieces was an estimate of those bacteria irreversibly attached to the surface.

Bacteria were enumerated for procedures a and b, above, by impedance microbiology (Bishop et al.,1985; Firstenberg-Eden and Tricarico, 1983), using a microbial monitoring system model 120 SC Bactometer, (Vitek Systems, St. Louis, Mo.). Modified plate count agar (MPCA), containing (in grams per liter) tryptone 20, yeast extract 20, agar 10 and dextrose 4, and modified plate count broth (MPCB) (same as MPCA without the agar), were used for the impedance test. Modules were filled by pipeting 0.6 ml of MPCA, allowing the agar solidify and then placing 0.9 ml of MPCB over the agar layer. Modules were prepared weekly, then placed in their initial individual bags, sealed and kept at refrigeration temperatures until use.

Pseudomonas fragi (Figure 1), was grown in NB overnight at 21° C and serial diluted from 10^{-1} to 10^{-8} in standard methods dairy diluent. The number of bacteria present in each dilution was evaluated by conventional methods, using Petrifilm Standard Method plates (Microbiology Products, Medical Products Division/3M, St. Paul, MN 55144). Each dilution was done in duplicate, and bacterial colonies were counted after 48 hours of growth, at 21° C. The serial dilutions of this bacterium were also inoculated in triplicate into bactometer modules, and detection times obtained. These detection times were then correlated to the respective numbers of bacteria obtained by conventional enumeration (figure 1). A detection time of 3 hours is approximately equivalent to a log cycle of bacteria. The conversion of detection times into numbers of bacteria was done using the mathematical equation obtained from linear regression analysis. Since the total area of each piece of buna-N-rubber was 70.398 mm^2 and that of teflon was 46.534 mm^2, the numbers of bacteria were further converted into numbers of bacteria per unit area).

c. The remaining three pieces of each gasket material were air dried, and prepared for direct epifluorescent microscopic viewing. After dried specimens were

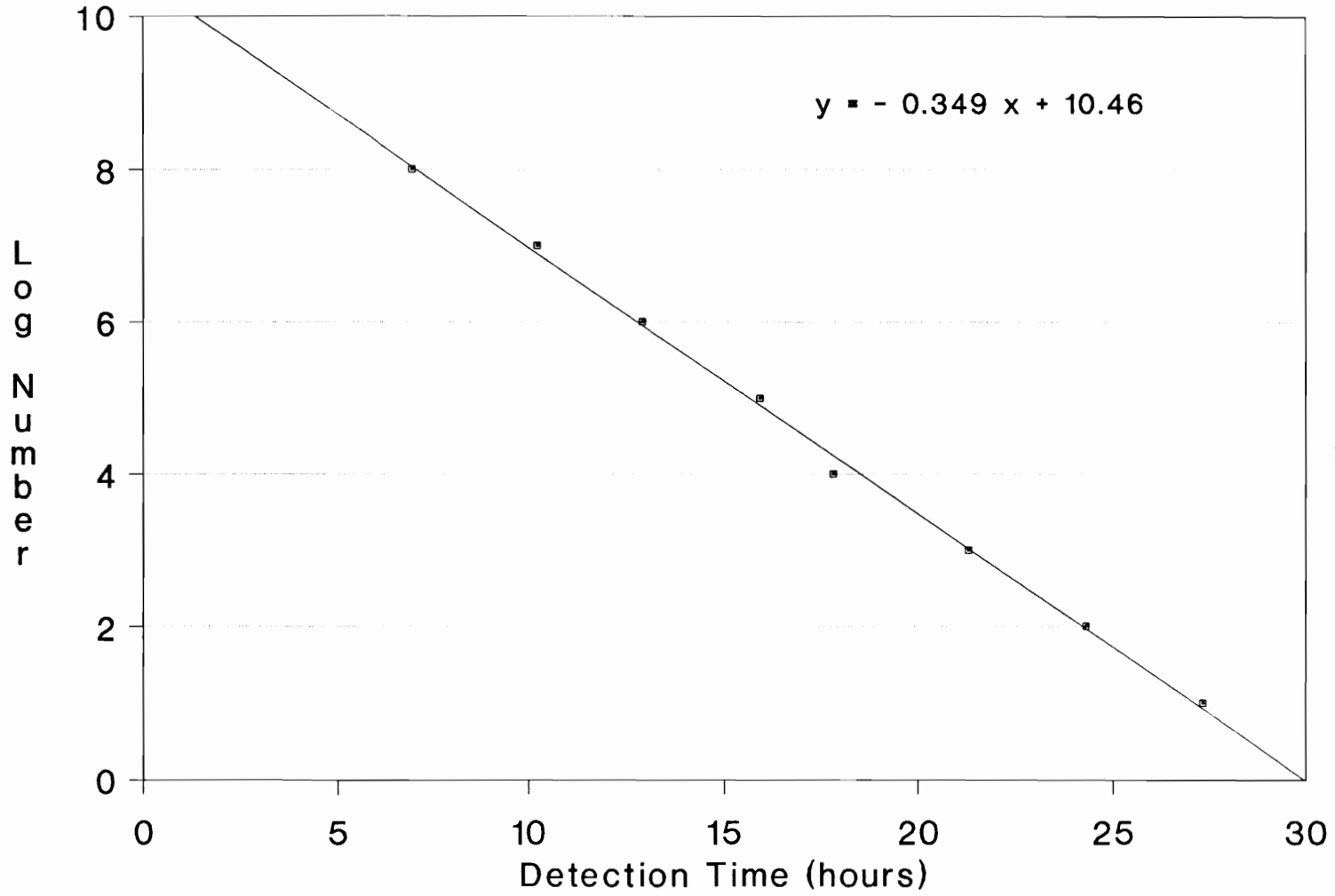

Figure 1. Standard curve for Pseudomonas fragi relating impedance detection times with numbers of bacteria in initial inoculum

fixed in Kirkpatrick's fixative for 3 minutes, they were drained and rinsed with ethyl alcohol and air dried. Specimens were exposed to 0.3% of Sentol for two minutes and rinsed with water, and stained with 0.03% fresh acridine orange for five minutes. Teflon pieces were then rinsed with water and air dried. Buna-N-rubber samples were rinsed with ethyl alcohol and air dried. Once dried, both types of samples were glued onto a glass slide (Czechowski,1989), and viewed on a Olympus BH-2 fluorescent microscope (Optical Elements Corporation, Washington, D.C.) using a 100x Plan D objective. This epifluorescent procedure was only qualitative since bacteria were impossible to count once the biofilm was formed.

D. Quantitative Analysis of the Affect of Nutrients on the Process of Bacterial Attachment

For this part of the study Pseudomonas fragi was used. Skim milk, casein, whey and casein with 4.9% lactose were used in varying dilutions: 1:1, 1:4; 1:32 (in distilled water). Skim milk was prepared by centrifugation of whole milk obtained from Virginia Tech dairy farm. The whole milk was centrifugated at 4,500xg for 30 minutes at 4° C. The skim milk was then removed and pasteurized in batches at 145° F for 30 minutes, kept at refrigeration temperature and used within a week of pasteurization. If the skim milk was to be used after one week storage it was steamed at 100°C for 10 min, and refrigerated.

To obtain the casein and whey, skim milk was ultra-centrifugated in a Beckman L5/50B ultracentrifuge at 100,000xg for one and a half hour at 4° C. The whey was removed and the casein pellet was resuspended in a salt solution (Jenness and Koop, 1962), to the initial volume of milk, and kept swirled gently until the casein pellet was totally resuspended. Both whey and resuspended casein were

steamed at 100°C for 10 minutes, and refrigerated until used. To some of the casein 4.9% of lactose was added, to maintain the concentration of lactose as in milk.

Skim milk, casein, whey, and casein with lactose were tested in triplicate, according to procedures previously described in section C.

V. Results and Discussion

A. Surface Analysis of Teflon and Buna N-Rubber

1. Scanning electron microscopy and X-ray analysis

The surface of buna-N-rubber appeared very irregular and rough, Plate 1. Plates 2 and 3 show the surface teflon which was generally smooth with some random rough areas. Plate 3 shows more detail of one of the rough areas.

Energy dispersive X-ray analysis of the surfaces of teflon and buna-N-rubber is shown in fig. 2 and 3, respectively. This analysis provides the elemental composition of the surfaces. Teflon (fig. 2) did not have elements other than fluorine. Buna-N-rubber (fig. 3), however, had calcium, silicone and sulfur. Teflon, tetrafluoroethylene, is made by polymerizing tetrafluoroethylene in an aqueous suspension and natural rubber is a 1,4-addition polymer of isoprene, (Solomons,1984).

Pure, natural rubber is soft and tacky, and has to be vulcanized to be useful. In vulcanization, natural rubber is heated with sulfur allowing for the production of cross-links between the poly-isoprene chains, which makes rubber much harder, (Solomons, 1984). The presence of silicone and calcium in buna-N-rubber probably represents materials added in the manufacturering process. Gristina (1987), analyzed the surface of biomaterials used in human surgery by energy dispersive x-ray analysis and found traces of iron, aluminum and other substances. He hypothesized that these trace materials might be due to the manufacture process or to impurities.

The presence of calcium in buna-N-rubber is inconclusive. Energy dispersive X-ray analysis identifies the elemental composition of the bulk of the sample. Further studies using electron spectrocopy (ESCA) should be conducted to determine if the most external layers of the surface have the same elemental composition determined by X-ray analysis.

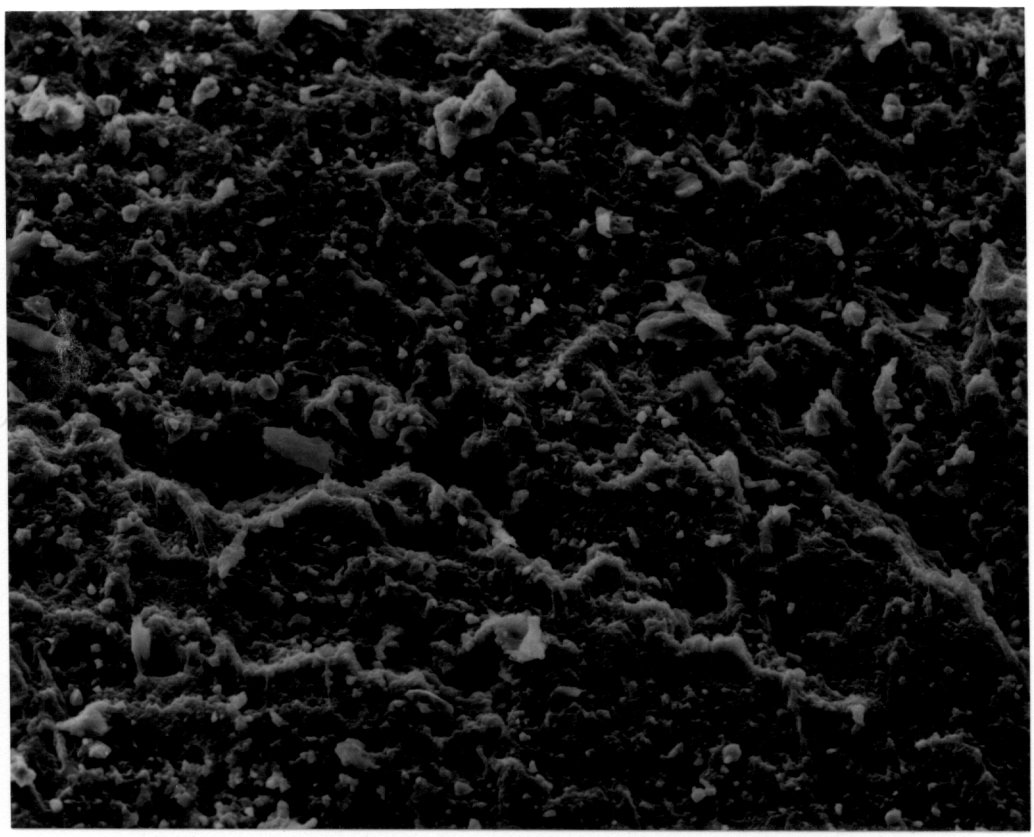

PLATE 1. Scanning electron micrograph of the surface of buna-N-rubber. Critical point dried sample (CPD). 420 x.

PLATE 2. Scanning electron micrograph of the surface of teflon. CPD. 470 x.

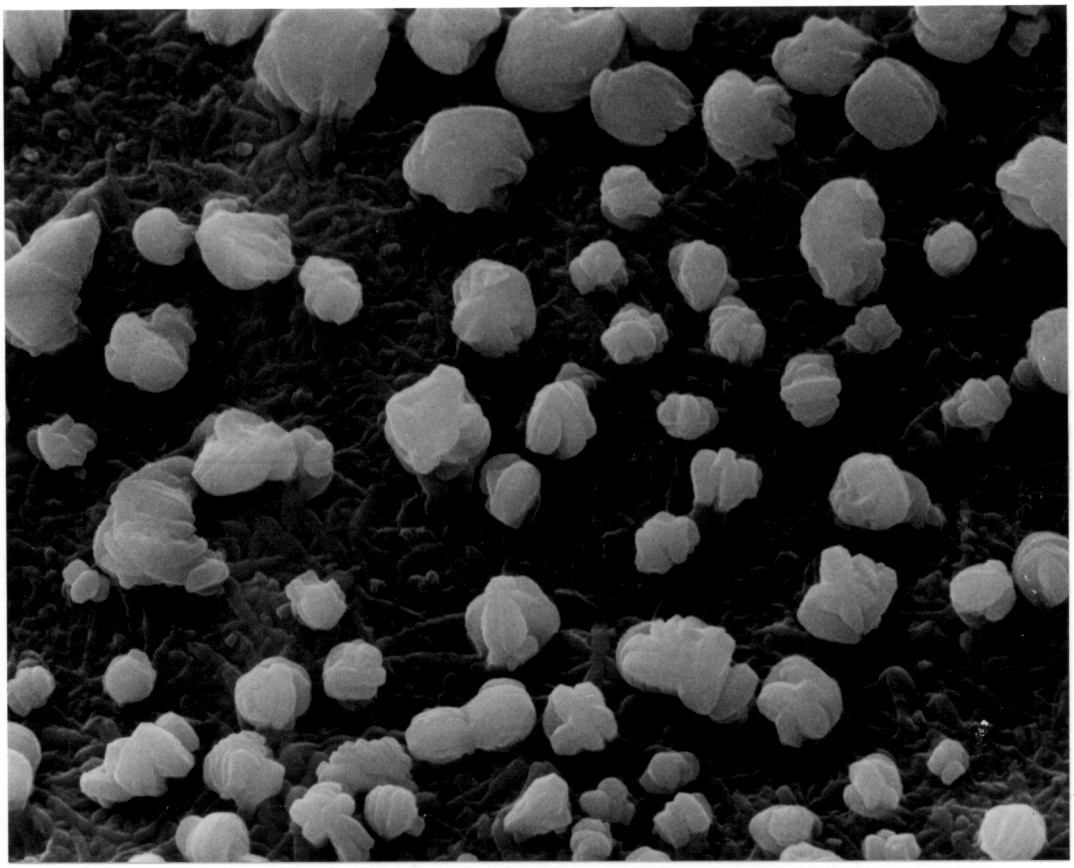

PLATE 3. Scanning electron micrograph of the rough area of teflon as shown in Plate 2. CPD. 3,800 x.

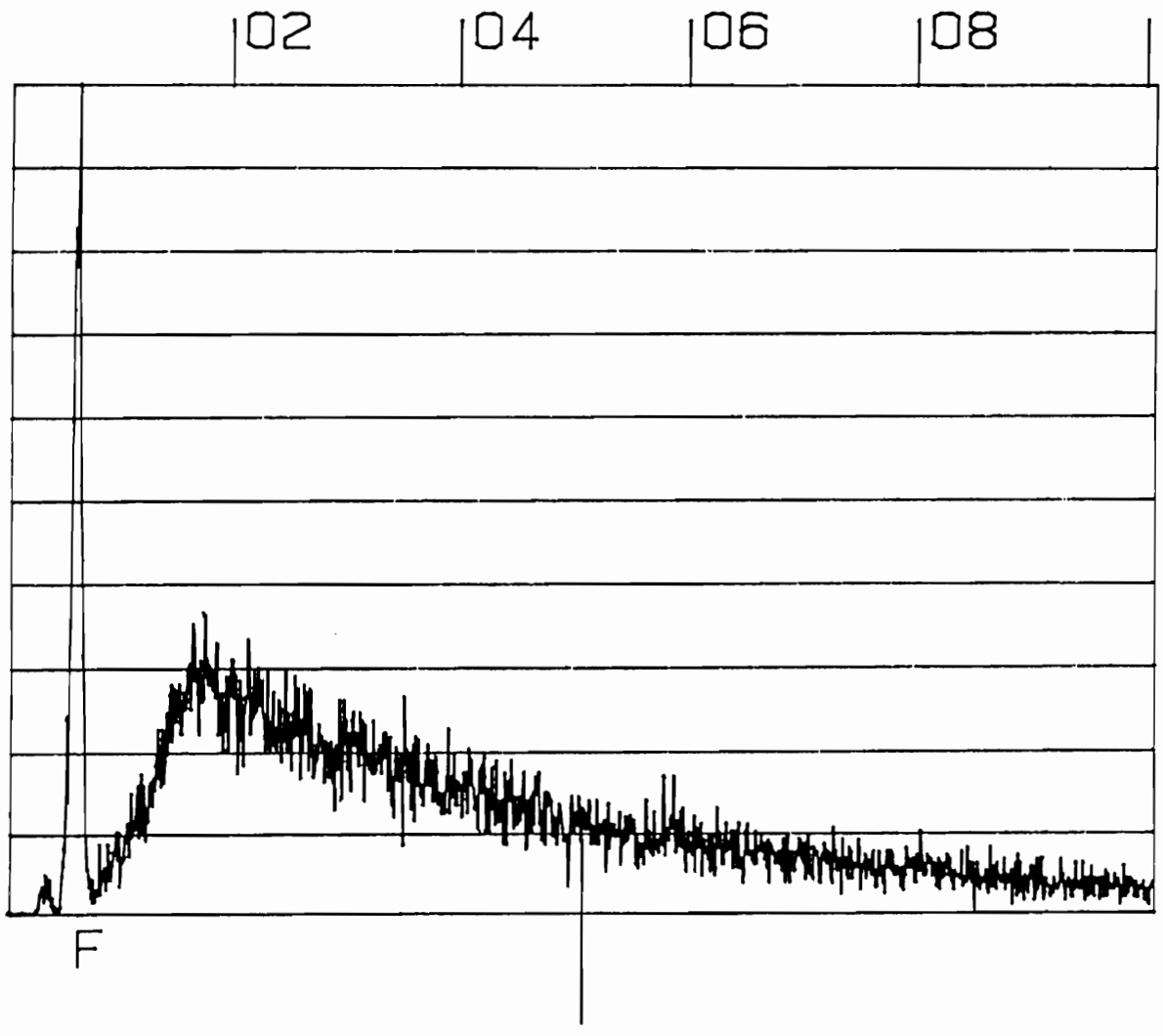

FIGURE 2. Energy dispersive analysis of X-rays emitted by teflon. Only fluorine (F) was identified.

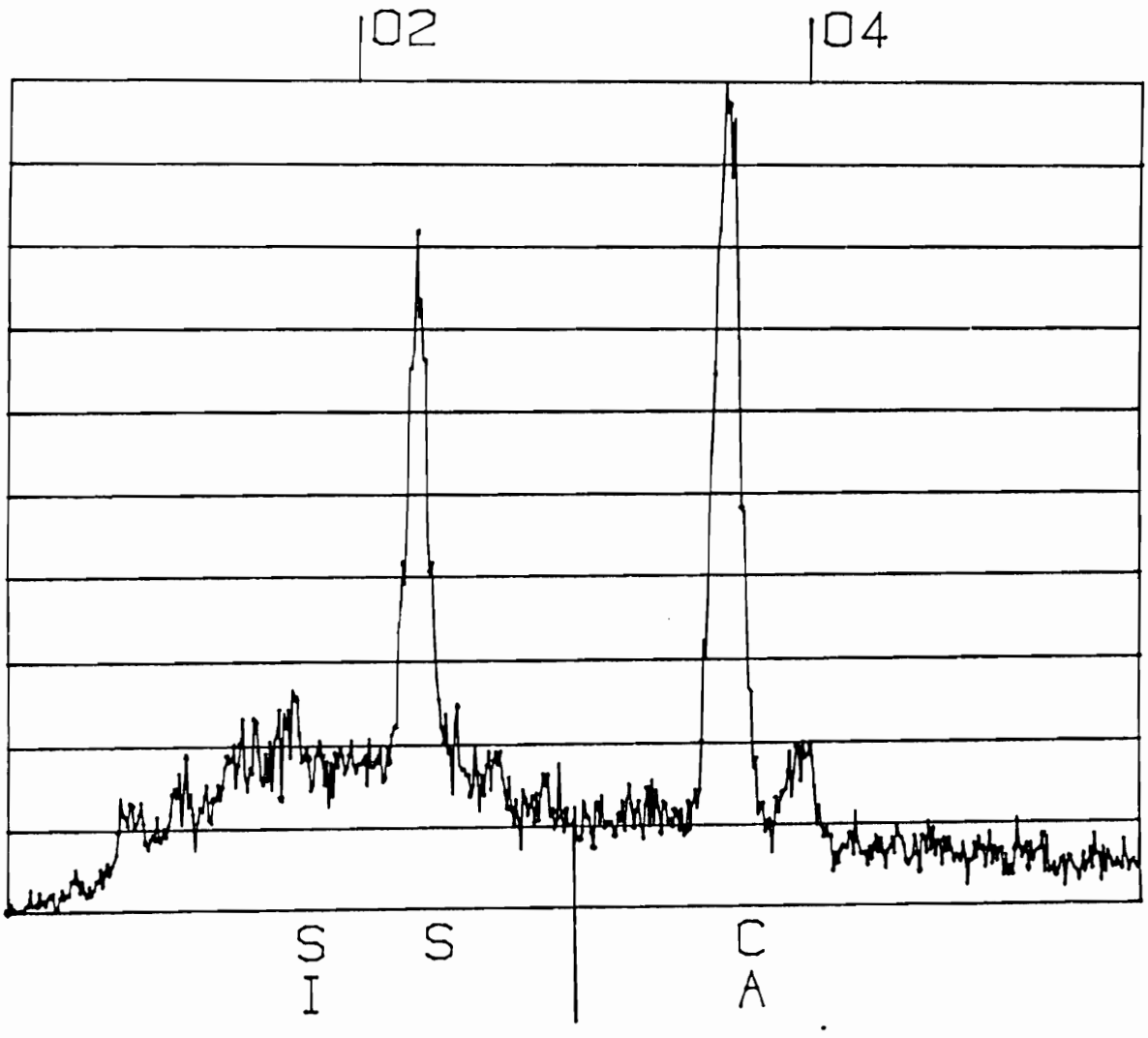

FIGURE 3. Energy dispersive analysis of X-rays emitted by buna-N-rubber . Silicone (Si), sulfur (S), and calcium (Ca) were identified.

1. Contact angle measurement

The water contact angle for teflon and buna-N-rubber was 112° and 151.1°, respectively. Good (1979), characterized solids as high-energy or low-energy on the basis of whether or not liquids with relatively high surface tension such as water, spread with zero contact angle on the solid. Contact angle values vary from 0° (surface with highest surface energy), to 180° (surface with zero surface energy). Buna-N-rubber has a lower surface energy with water contact angle of 151.5°, when compared to teflon which has a water contact angle 112°. Buna-N-rubber is a more hydrophobic surface than teflon.

Baier (1973, 1980) found bacterial adhesion to be minimal when substrata are relatively hydrophobic and this results were confirmed by Dexter (1979) and Dexter et al. (1975). Others (Carson and Allsopp, 1980; Fletcher and Loeb, 1979, 1976; Loeb 1977) found bacteria to attach in higher numbers to more hydrophobic surfaces. Our results agree with those of the latter group of researchers, Pseudomonas fragi attached in significantly higher numbers to buna-N-rubber than to teflon.

B. Qualitative Analysis of Bacterial Attachment to Teflon and to Buna-N-Rubber Surfaces

Teflon and buna-N-rubber were exposed to uninoculated milk. Scanning electron micrographs were taken of the milk film covering teflon for samples that were critical point dried (Plates 4 and 5). The difference between the two micrographs was very significant, the freeze-dried sample had a comparatively thicker and less disrupted film than the critical point dried sample. This was due to the better preservation of the three dimensional structure of the milk film when frezze-drying was used. Critical point dried samples are first dehydrated on a series of

alcohols and this destroys the structure of the milk film. The structure of the milk film on buna-N-rubber, from a freeze dried sample (Plate 6), was different than the one observed on teflon, suggesting a different type of interaction between milk molecules and each of the two surfaces studied.

All three bacteria studied readily attached to both teflon and to buna-N-rubber (Plates 7 to 24). Pseudomonas fragi attached to either surface within 2 hours of exposure (Plate 7). Plates 7 to 13 show the attachment of Pseudomonas fragi to both surfaces . At 4 hours of exposure (Plate 8), numerous cells were present on teflon, and some attachment fibrils were visible . These fibrils were not seen at 2 hours of bacterial growth (Plate 7). Plates 9, 10, and 11 represent attachment of Pseudomonas fragi at 6 hours of exposure, more bacteria seem to be present on both surfaces and attaching fibrils were more evident. On plate 9 and 10 a layer of material covering teflon is apparent, and bacteria are anchored on that layer.

Meadows (1971), Fletcher (1976) and Orstavik (1977), reported that bacterial adhesion to surfaces may be enhanced, inhibited or unaffected by the presence of macromolecule in the aqueous phase, and Maroudas (1980), suggested that there was macromolecule adsorbption to the surface of the substratum, thus altering the initial surface properties. The effect of the adsorption of macromolecules of milk to teflon and buna-N-rubber surfaces in the process of bacterial attachment needs to be investigated.

Plate 11 shows the attachment of Pseudomonas fragi to buna-N-rubber at 6 hours of exposure. Attaching fibrils are easily seen and bacteria seem to be located in a crevice of the surface. At 48 hours of exposure (Plate 12), extra-cellular polymeric fibrillar "webbing" (Paerl,1985), is visible on teflon. This extracellular polymeric material bridges bacteria to the surface and also ensures bacterial aggre-

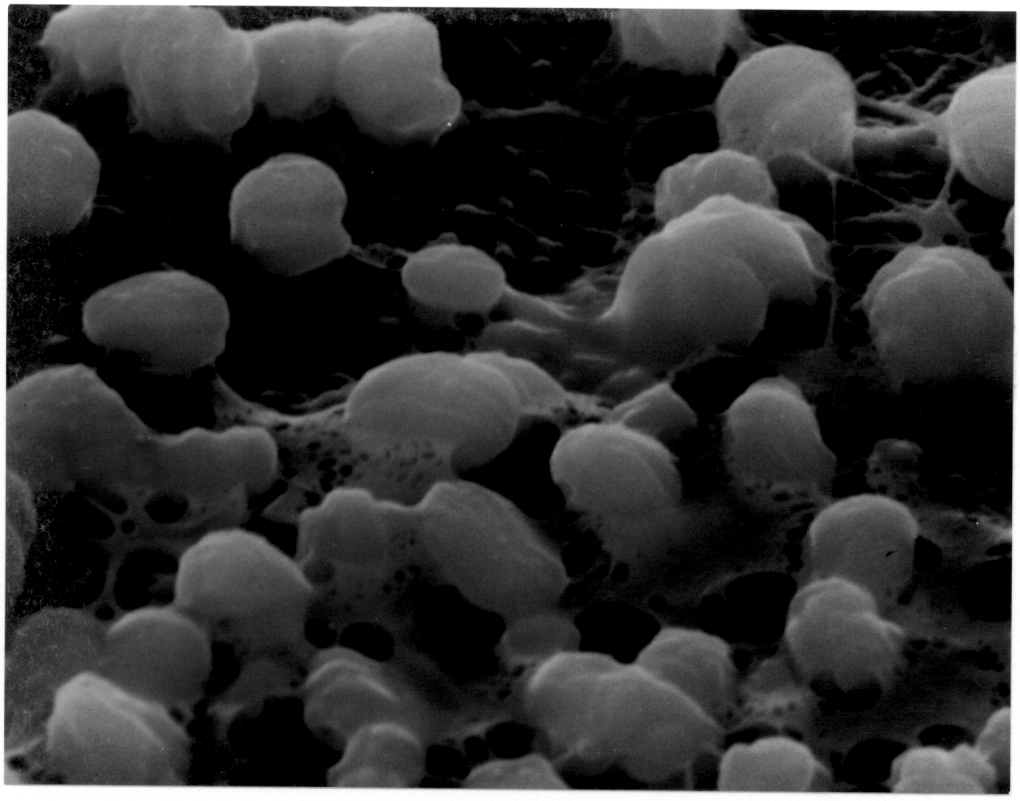

PLATE 4. Scanning electron micrograph of milk film on teflon after 6 hours of exposure. CPD. 7,000 x.

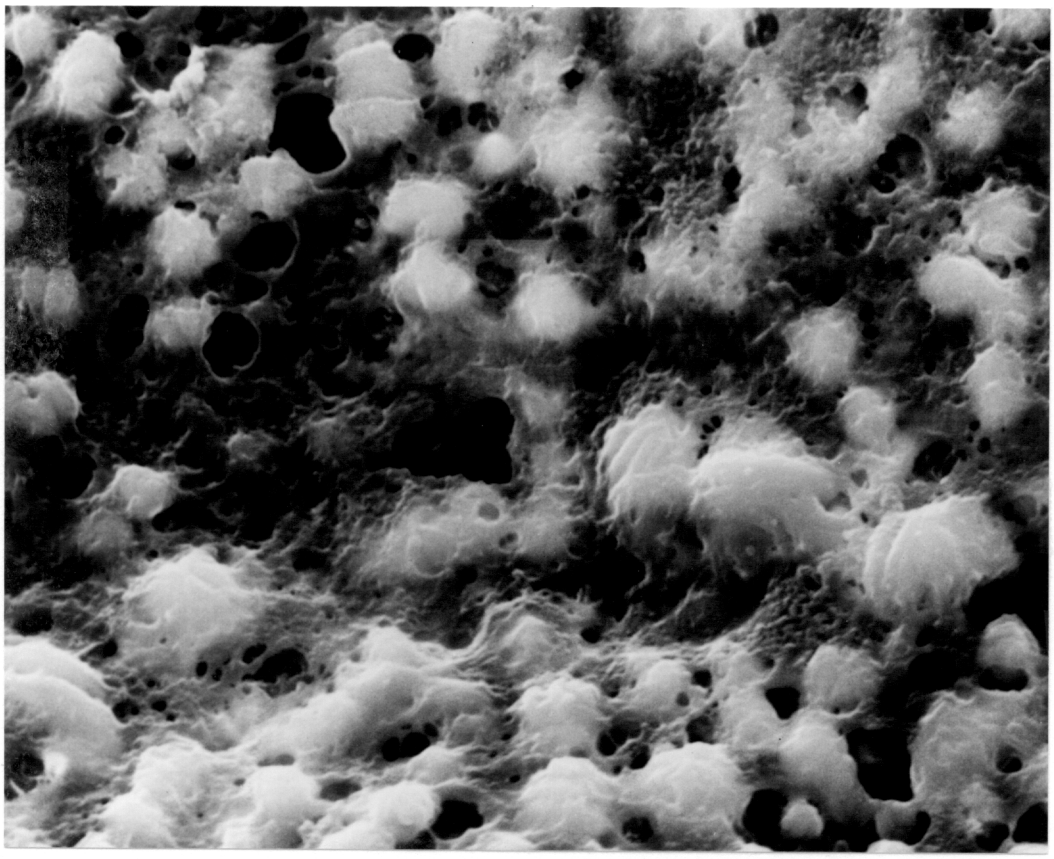

PLATE 5. Scanning electron micrograph of milk film on teflon after 6 hours of exposure. Freeze dried sample (FD). 3,800 x.

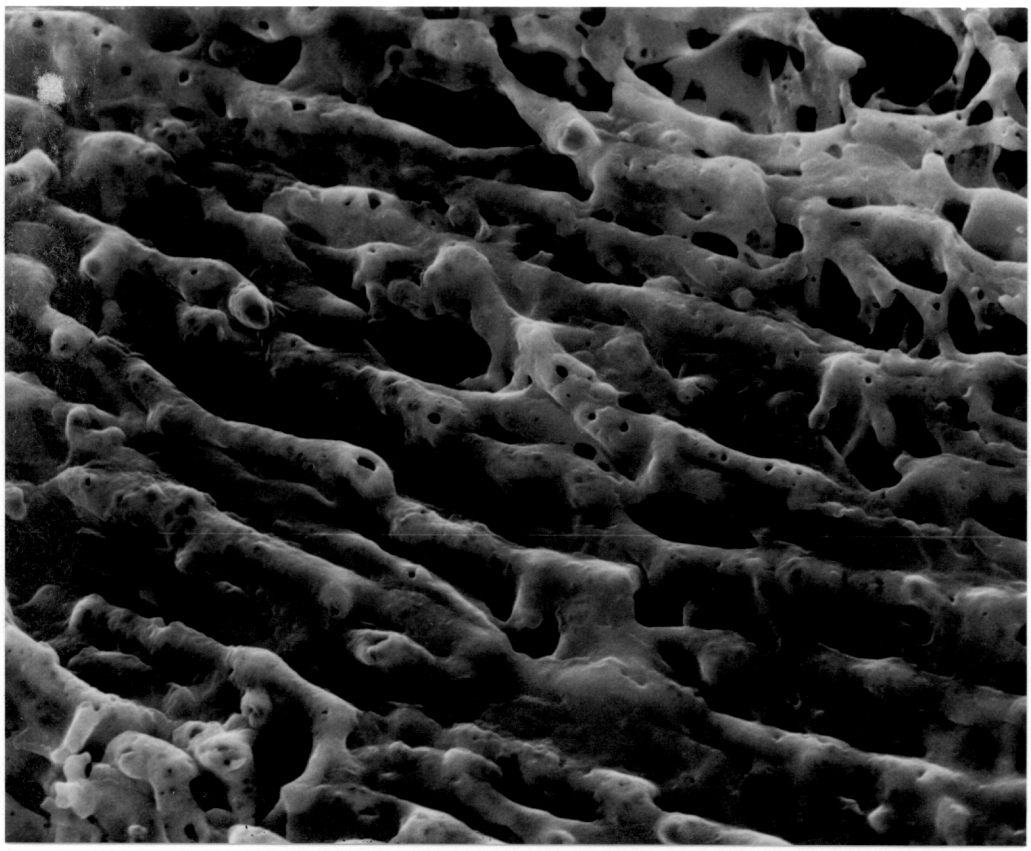

PLATE 6. Scanning electron micrograph of milk film on buna-N-rubber after 72 hours of exposure. FD. 2,000 x.

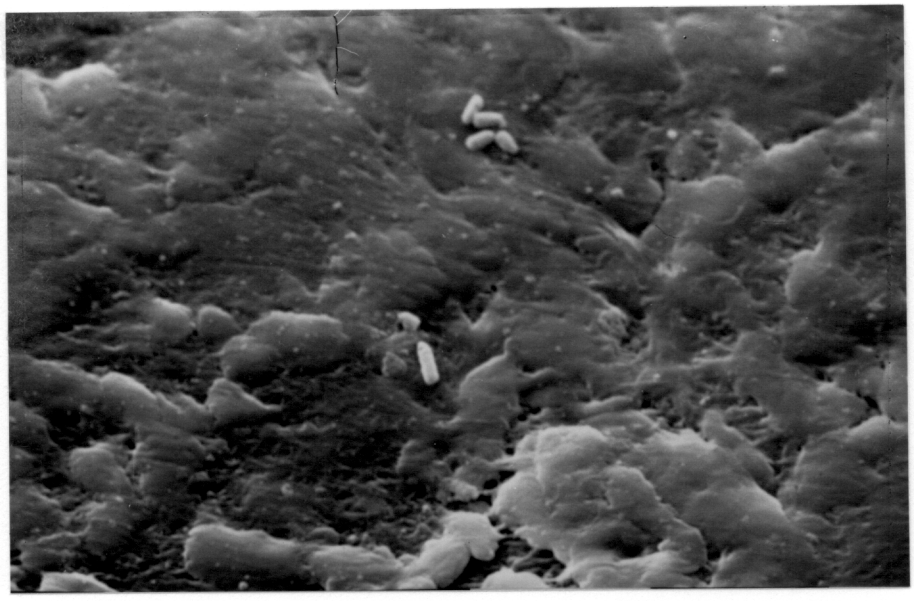

PLATE 7. Scanning electron micrograph of Pseudomonas fragi on teflon, 2 hours of exposure. CPD. 2,500 x.

PLATE 8. Scanning electron micrograph of Pseudomonas fragi on teflon, 4 hours of exposure. Attaching fibrils are visible. CPD. 2,700 x.

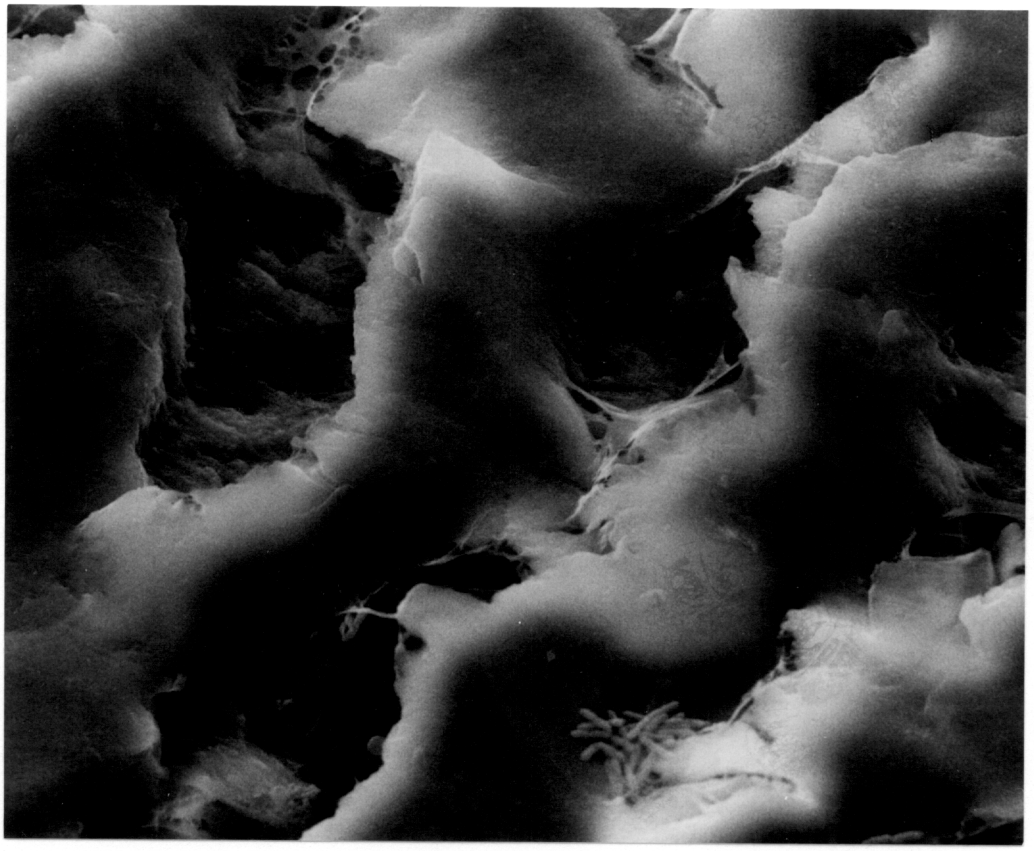

PLATE 9. Scanning electron micrograph of Pseudomonas fragi on teflon, 6 hours of exposure. Bacteria are lying on a layer of milk. CPD. 1,300 x.

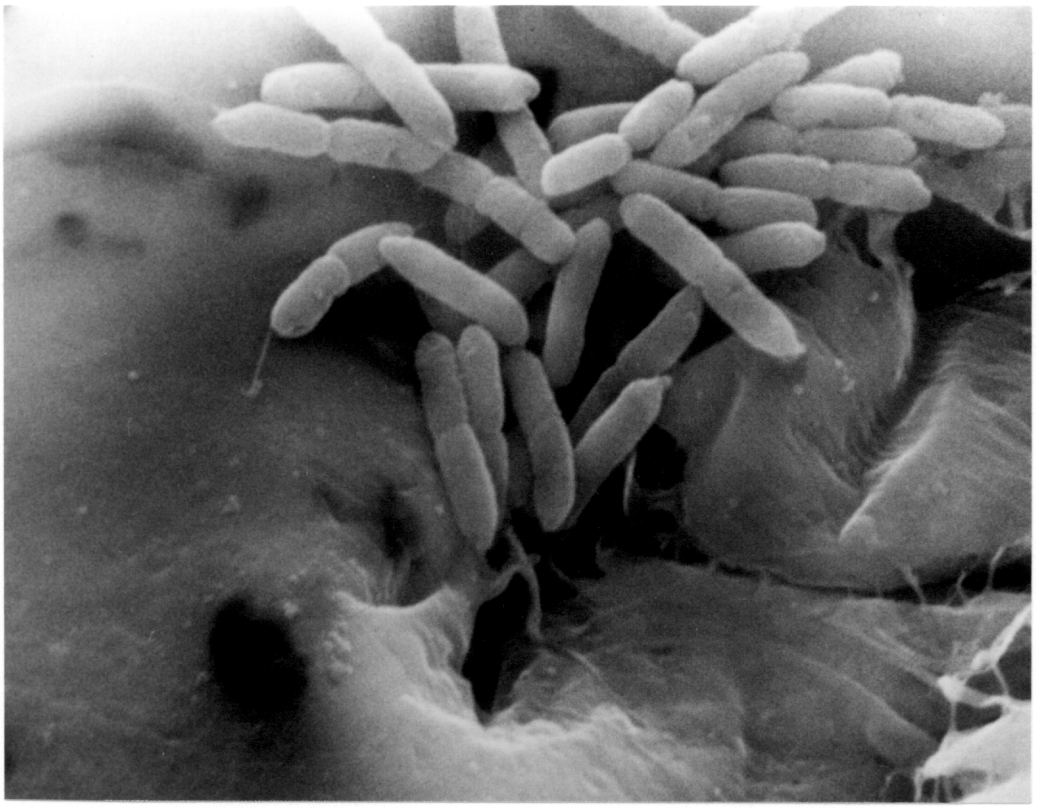

PLATE 10. Scanning electron micrograph of Pseudomonas fragi on teflon, detail of Plate 9. Attaching fibrils are visible. 7,400 x.

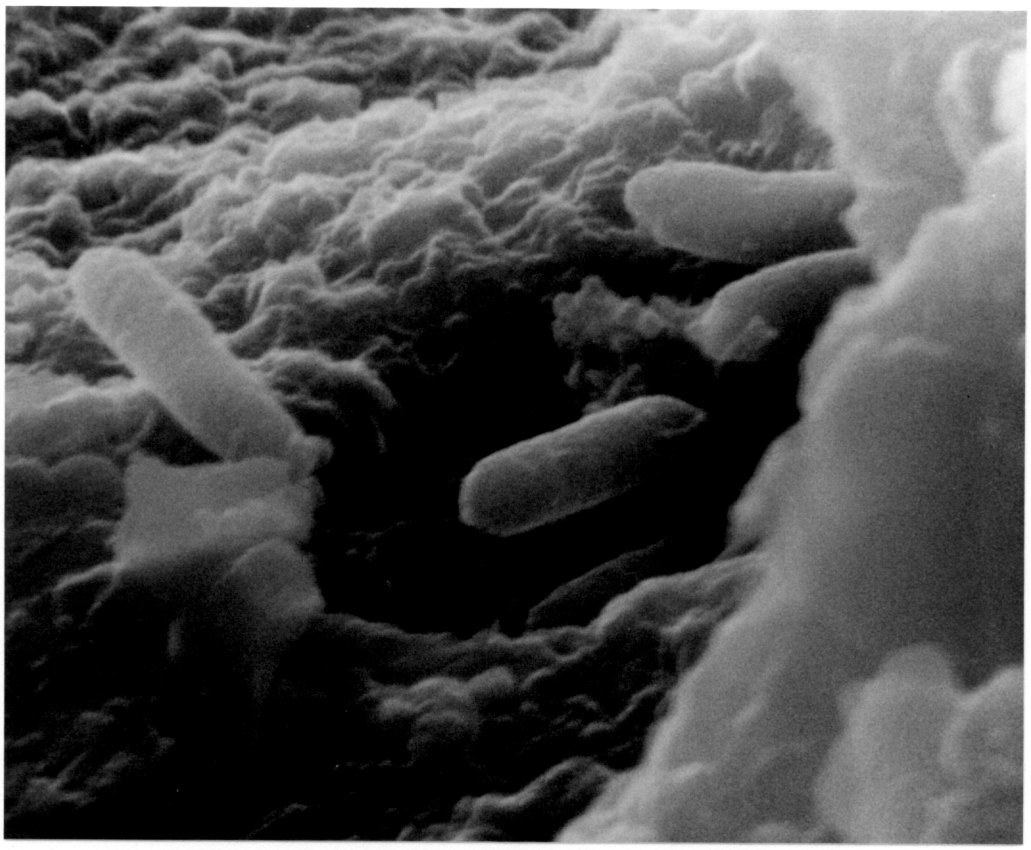

PLATE 11. Scanning electron micrograph of Pseudomonas fragi on buna-N-rubber, 6 hours of exposure. Attaching fibrils are visible. CPD. 15,000 x.

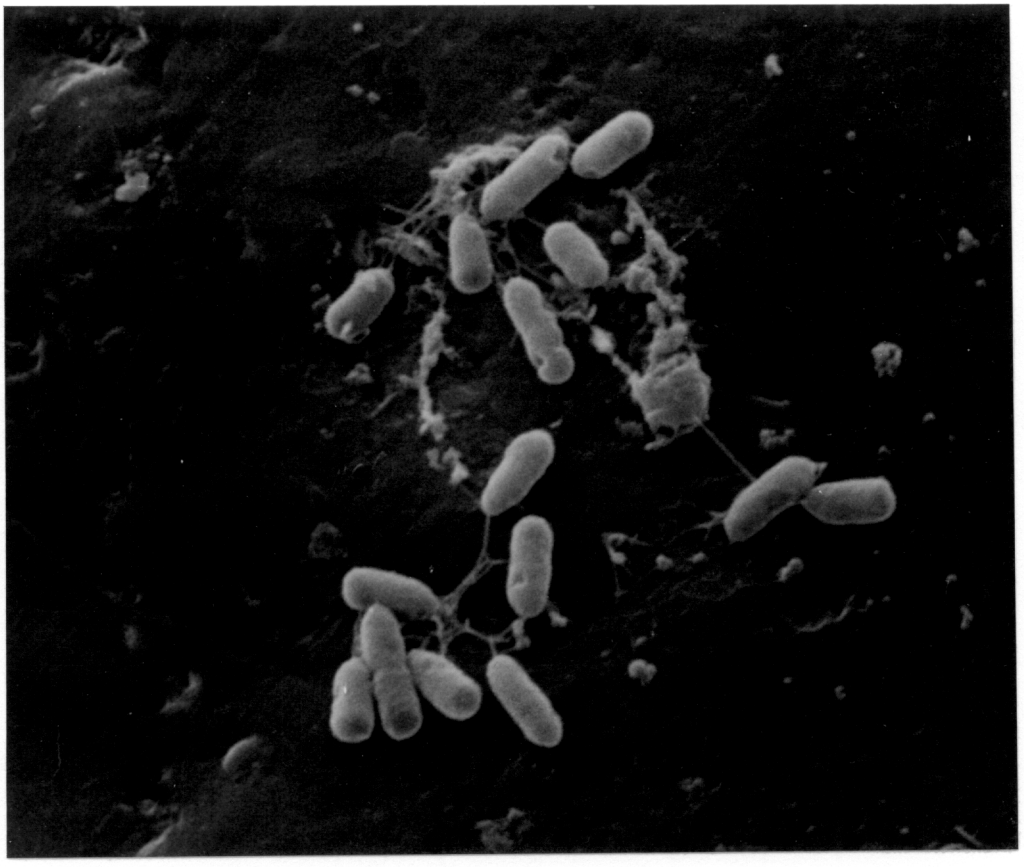

PLATE 12. Scanning electron micrograph of Pseudomonas fragi on teflon, 48 hours of exposure. Attaching fibrils are numerous. CPD. 7,300 x.

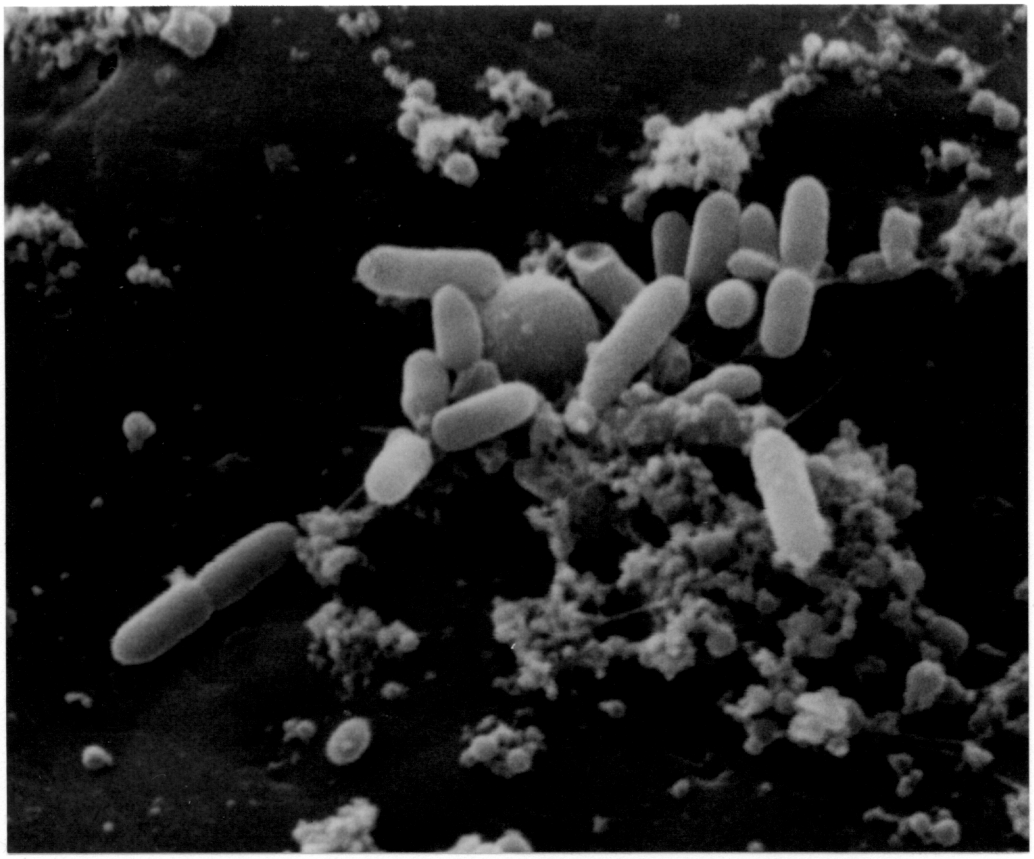

PLATE 13. Scanning electron micrograph of Pseudomonas fragi on teflon, 72 hours of exposure. Attaching fibrils are numerous. CPD. 8,000 x.

gation (Marshall,1971;Paerl,1985; Costerton,1981;). Plate 13 shows the attachment of Pseudomonas fragi at 72 hours of exposure. The extracellular polymeric fibrillar material is evident linking bacteria between each other and to the surface, entrapping some other materials. We hypothesize to be bacterial metabolic by-products and polymeric material of the biofilm.

Marshall et al. (1971a), postulated bacterial attachment to solid surfaces to be a two step process. The first phase is a reversible due to non-specific general long-range forces, and the second phase is an irreversible time dependent process due to polymer bridging between the bacteria and the surface. Firstenberg-Eden et al. (1979) also found bacterial attachment to the surface of teats of cows to be a two-step process with extra-cellular polymer formation appearing 2-3 hours after contamination. Zoltai et al. (1981), reported fibrous appendages within 6 hours of bacterial growth on stainless steel. Our results agree with those, and a sequential process of attachment Pseudomonas fragi to both buna-N-rubber and teflon is suggested by the sequence of the micrographs obtained. The amount of attaching fibrillar material increased with time of exposure.

Attachment of Bacillus cereus to the surfaces of both materials was first seen after 12 hours of exposure (Plate 14). The initial inoculum for Bacillus cereus had 100x less bacteria per mililiter than the one used for the previous organism and this experiment was performed at 21°C. The best growth temperature for this organism is 30°C, and this fact complemented with the smaller initial inoculum used, may have caused the initial growth rate to be slow, allowing for a comparatively smaller number of bacteria to be present in the liquid phase after 12 hours of bacterial growth. This could have delayed the time for the initial biofilm formation in comparison to Pseudomonas fragi (Bryers and Characklis, 1981). In Plate 14,

PLATE 14. Scanning electron micrograph of Bacillus cereus on teflon, 12 hours of exposure. Attaching fibrils are visible. CPD. 2,100 x.

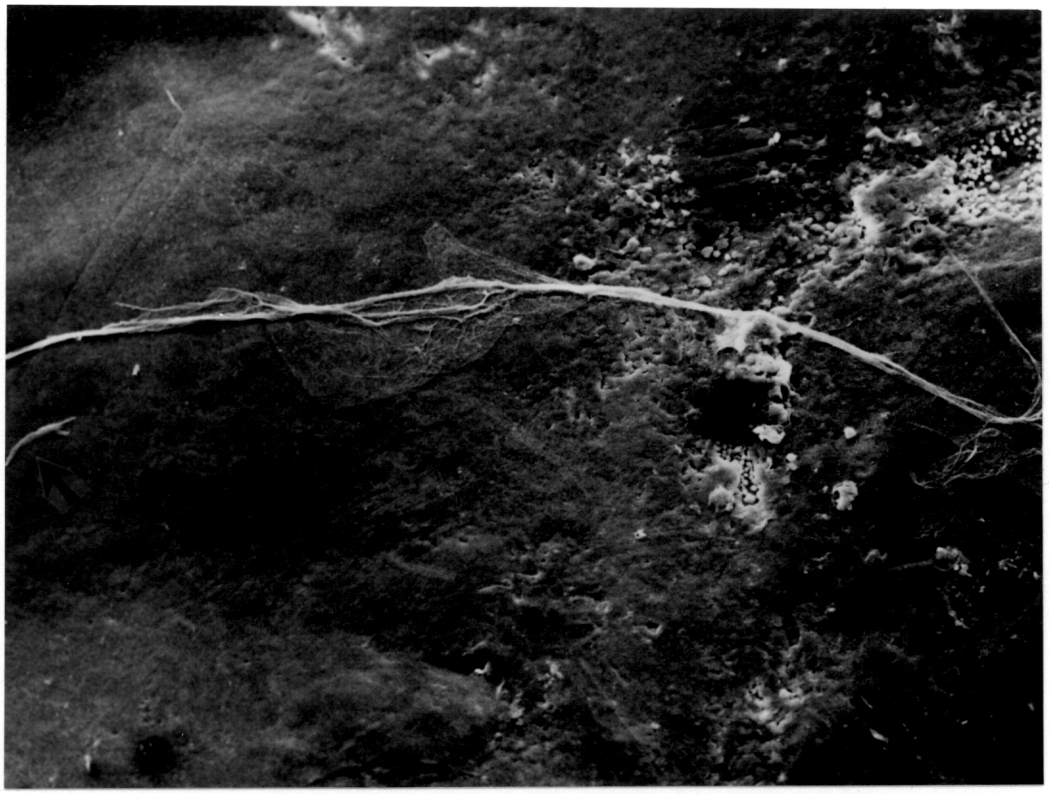

PLATE 15. Scanning electron micrograph of Bacillus cereus on teflon, 24 hours of exposure. Bacterial colony identified with arrow is enlarged on Plate 16. CPD. 260 x.

extracellular attachment polymeric material appears to be present. This differs from the fibrils seen with Pseudomonas fragi. Plate 15 shows bacterial attachment of this bacterium to teflon at 24 hours of exposure. At this time of exposure numerous colonies were visible on both surfaces. This specific colony appeared "hairy" like, spreading over a large area of the surface. A small field of this micrograph was magnified (Plate 16). Morphologically, the microorganism was elongated, possibly due to the fact that they were growing at a very high rate, and "footprints" are also visible (Marshall et al.,1981a). Plate 17 also from 24 hours of exposure, shows the microorganism still elongated and it had a "slimy" appearance with some visible attaching materials. After 48 hours of exposure on teflon (Plate 18), a fairly well preserved layer of compounds is present which appear to protect bacteria in some areas. This protective coating resisted the alcohol dehydration process fairly well, allowing for a better understanding of the biolfilm structure. In some parts of the micrograph bacteria can be seen buried under the protective coating, possibly formed by bacterial exopolysaccharides and other molecules such as bacterial by-products. After 72 hours of exposure (Plate 19), there was an intact protective coating that covered the bacteria cells, this sample was freeze-dried. In some regions the protective coating is broken, possibly due to shrinkage of the biological material during dehydration (Bessis and Weed,1972). After 72 hours of exposure of buna-N-rubber (Plates 20 and 21) biofilm formation was similar to that seen with teflon with the protective coating still visible in some areas and in other areas bacteria seem "bare".

The results obtained are slightly different than some previous reports on attachment of various other gram-positive bacteria. Firstenberg-Eden et al. (1979) found presence of Staphylococcus aureus on the surface of teats of cows,

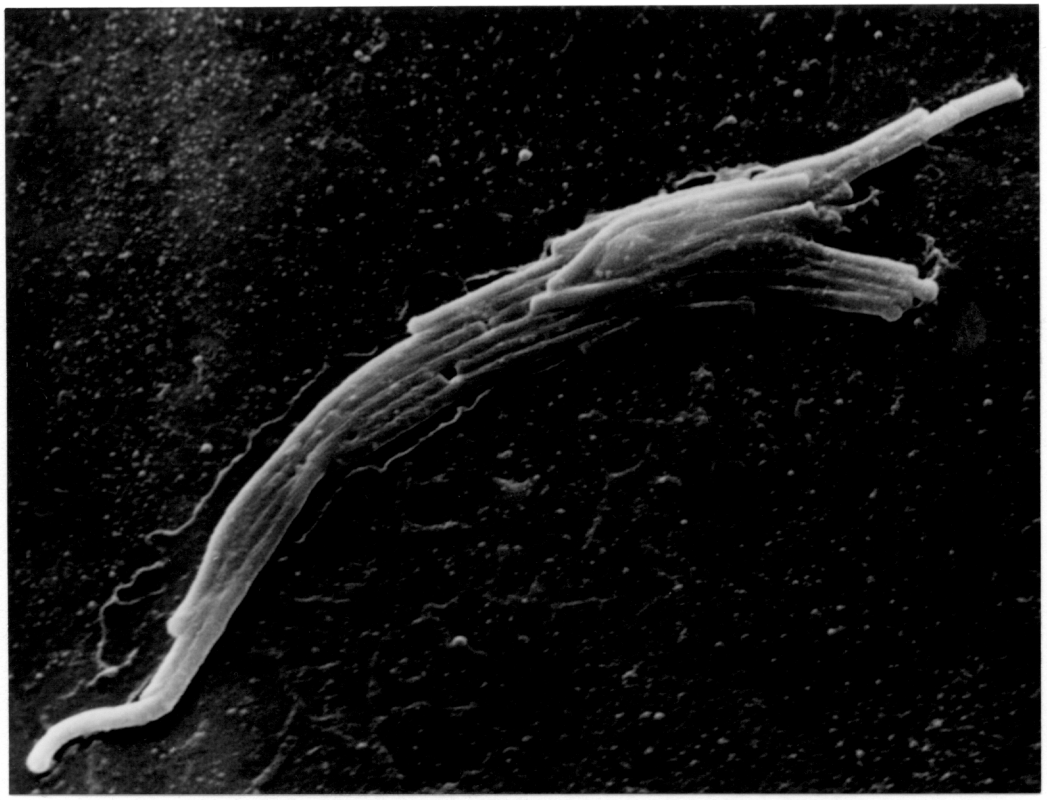

PLATE 16. Scanning electron micrograph of Bacillus cereus on teflon, 24 hours of exposure. Detail of previous micrograph. CPD. 3,900 x.

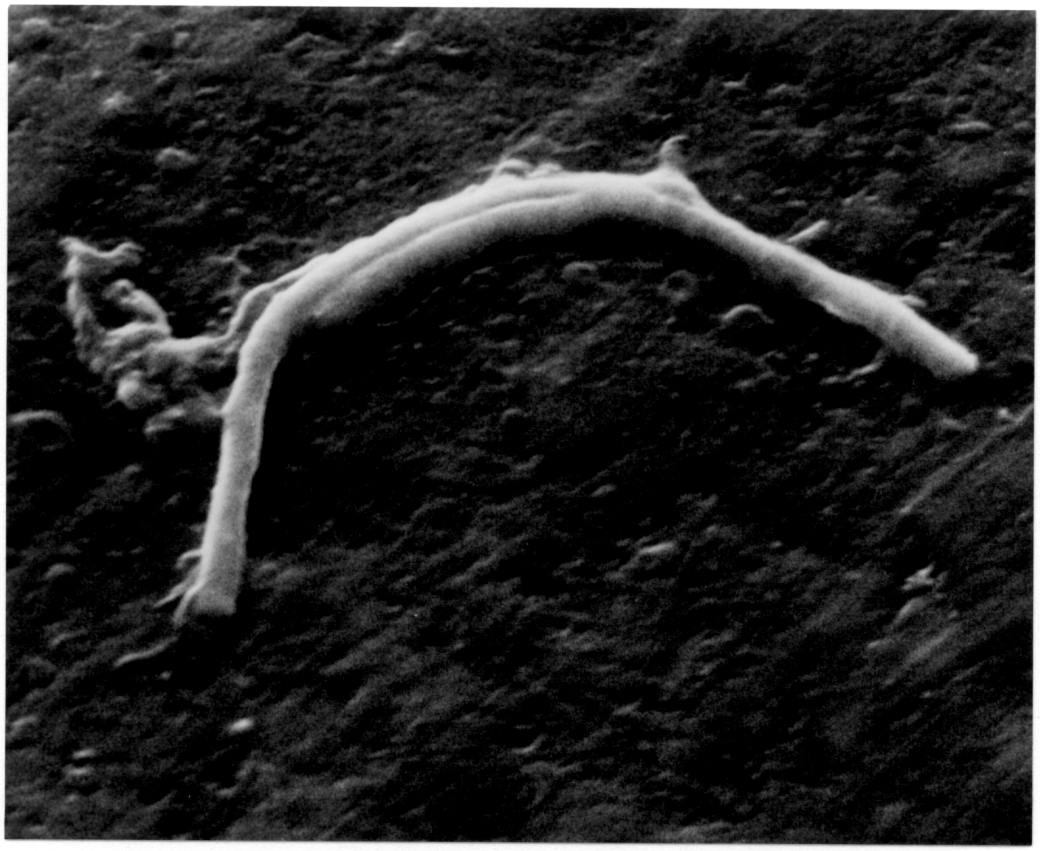

PLATE 17. Scanning electron micrograph of Bacillus cereus on teflon, 24 hours of exposure. Attaching fibrils are visible. CPD. 4,700 x.

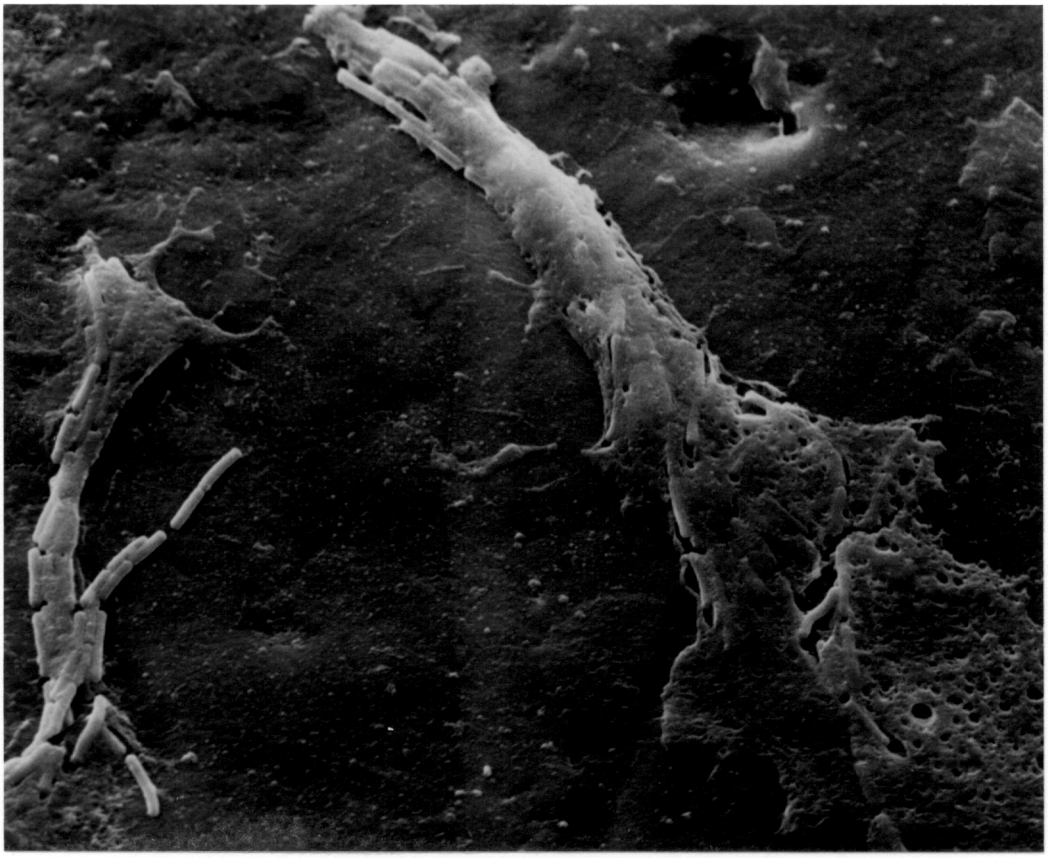

PLATE 18. Scanning electron micrograph of Bacillus cereus on teflon, 48 hours of exposure. In some areas bacteria are covered with the protective layer of the biofilm. CPD. 1,400 x.

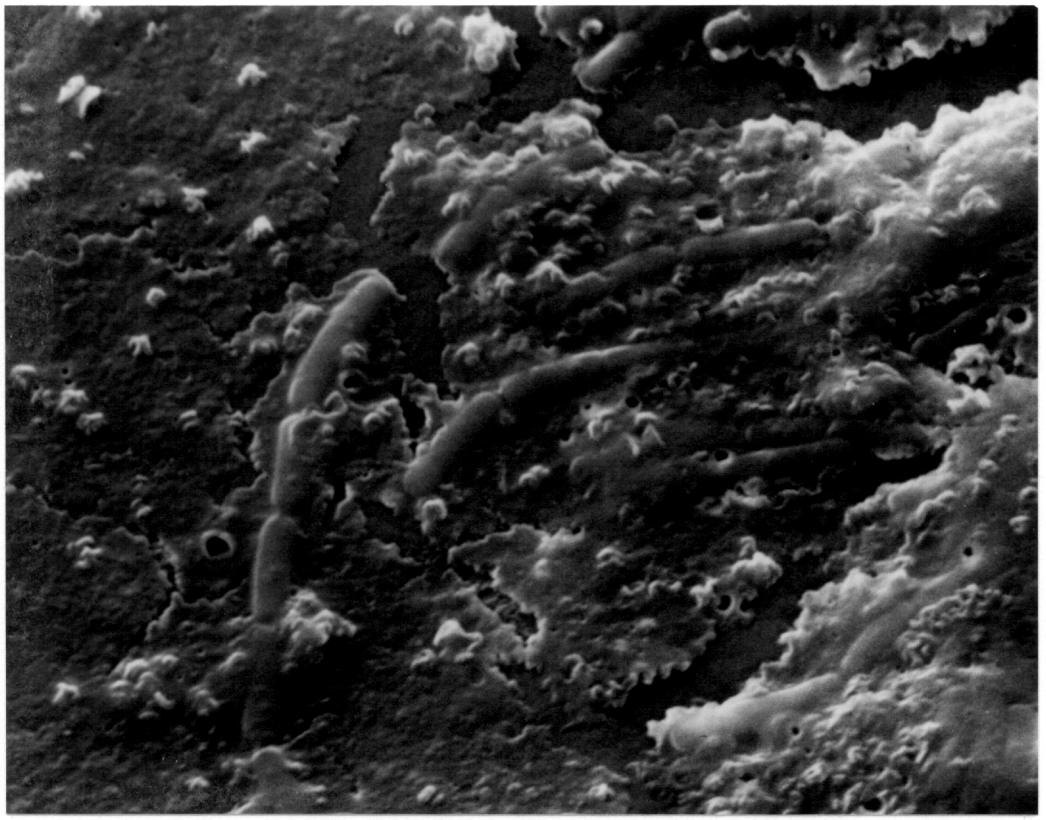

PLATE 19. Scanning electron micrograph of Bacillus cereus on teflon, 72 hours of exposure. FD. 3,400 x.

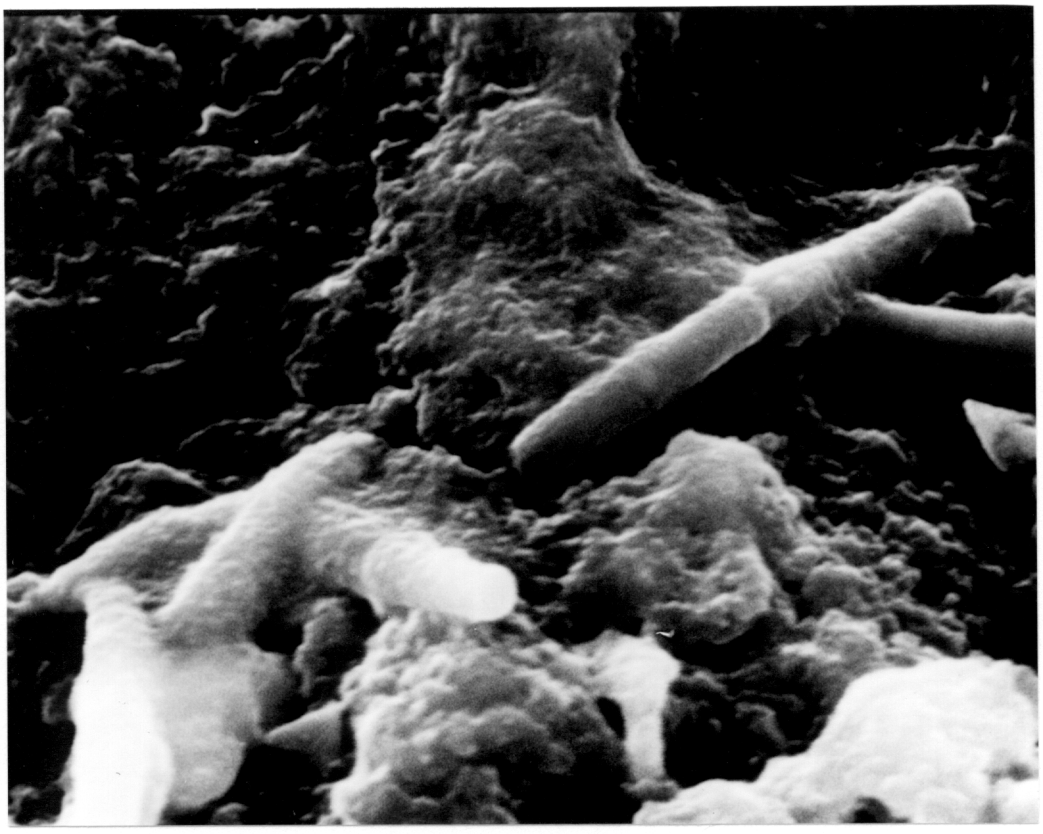

PLATE 20. Scanning electron micrograph of Bacillus cereus on buna-N-rubber, 72 hours of exposure. Bacteria are partially covered with the protective coating of the biofilm. CPD. 7,500 x.

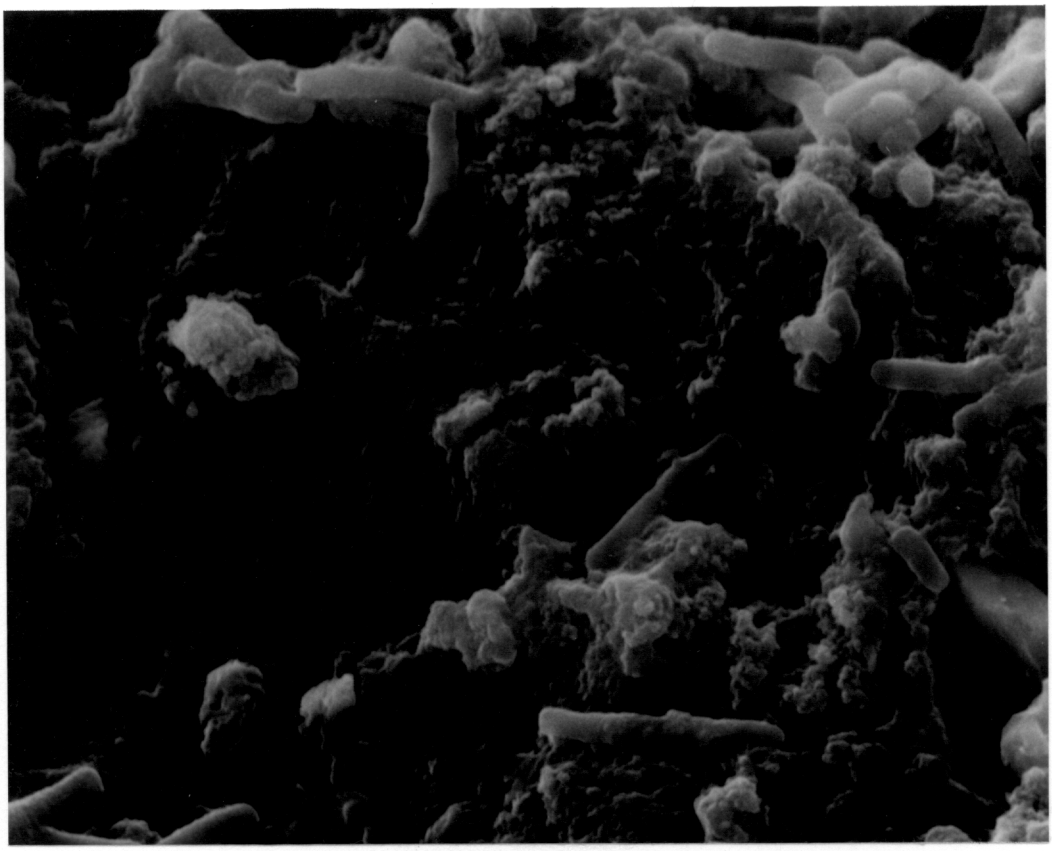

PLATE 21. Scanning electron micrograph of Bacillus cereus on buna-N-rubber, 72 hours of exposure. CPD. 4,500 x.

cows, immediately after contamination. Buttler et al. (1979) found Lactobacillus sp. to attach to beef and lamb skin samples after 1 minute of exposure of the skin to the attaching suspension while Zoltai et al. (1981) reported that for Staphylococcus aureus and Lactobacillus bulgaricus RR, slime was not evident after 12 hours of contact time. The micrographs obtained for Bacillus cereus suggest a sequential process of bacterial attachment to the two surface studied, with an increase in the amount of extra-cellular polymeric material detected as time of exposure of both surfaces progressed along.

For Listeria monocytogenes, bacterial cells were detected on both surfaces only at 24 hours of bacterial exposure (Plates 22 to 24). Plate 22, shows the attachment of this bacterium to teflon after 24 hours of bacterial exposure; the attaching fibrils are evident. At 48 hours of bacterial exposure (Plate 23), the biofilm on teflon is clearly visible, with the organisms appearing in different layers and mixed with some materials. These materials are probably bacterial cellular exopolysaccharides and molecules from products of bacterial metabolism. Plate 24 shows Listeria monocytogenes on buna-N-rubber after 48 hours of bacterial exposure This picture was very similar to that obtained for teflon. No flagella were seen at any time of bacterial growth. Herald and Zottola (1988) reported the presence of flagella when this bacterium attached to stainless steel, with growth temperatures of 35°C, 21°C and 10°C. The same authors also suggested that some isolated cells were visible on the surface at 4 and 8 hours of bacterial growth. Mafu et al. (1990) found attachment of Listeria monocytogenes to various solid surfaces after contact times of 20 minutes; however, both research groups used a much gentler rinsing process to remove bacteria reversibly attached to the surfaces and this fact could perhaps explain the differences in the results obtained. The sequence of these micrographs

PLATE 22. Scanning electron micrograph of Listeria monocytogenes on teflon, 24 hours of exposure. Attaching fibrils are seen. CPD. 5,300 x.

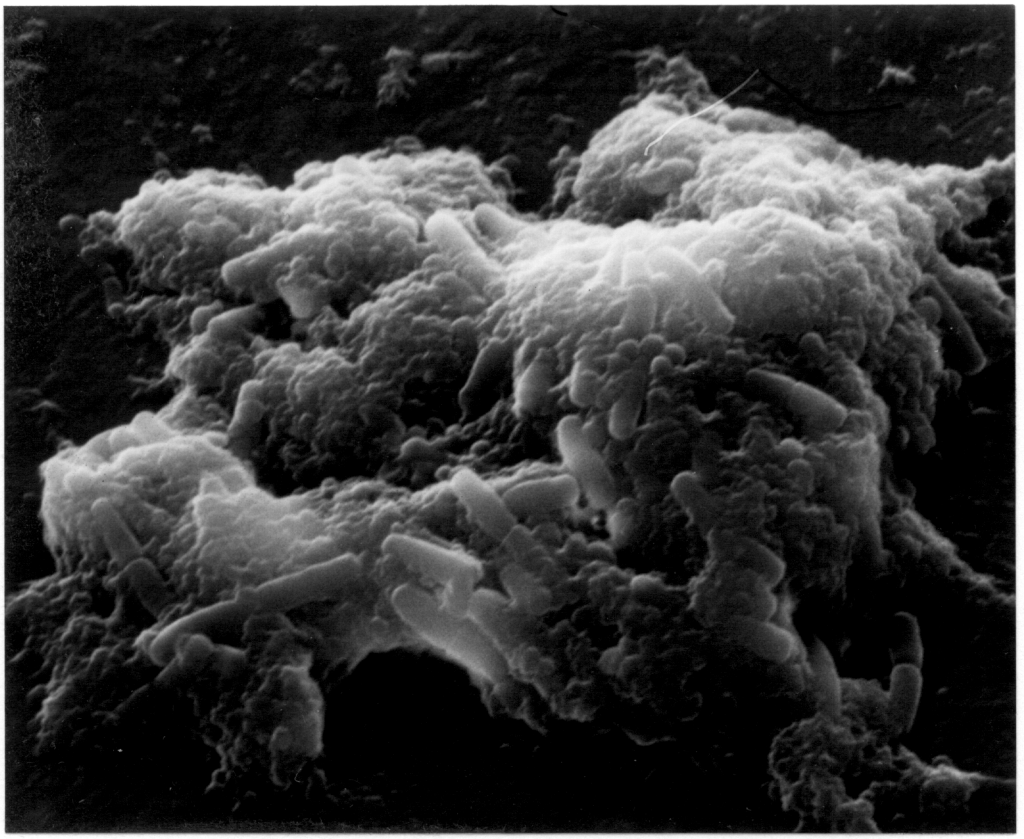

PLATE 23. Scanning electron micrograph of Listeria monocytogenes on teflon, 48 hours of exposure. Biofilm is evident with bacterial cells dispersed in a mass of material. CPD. 3,700 x.

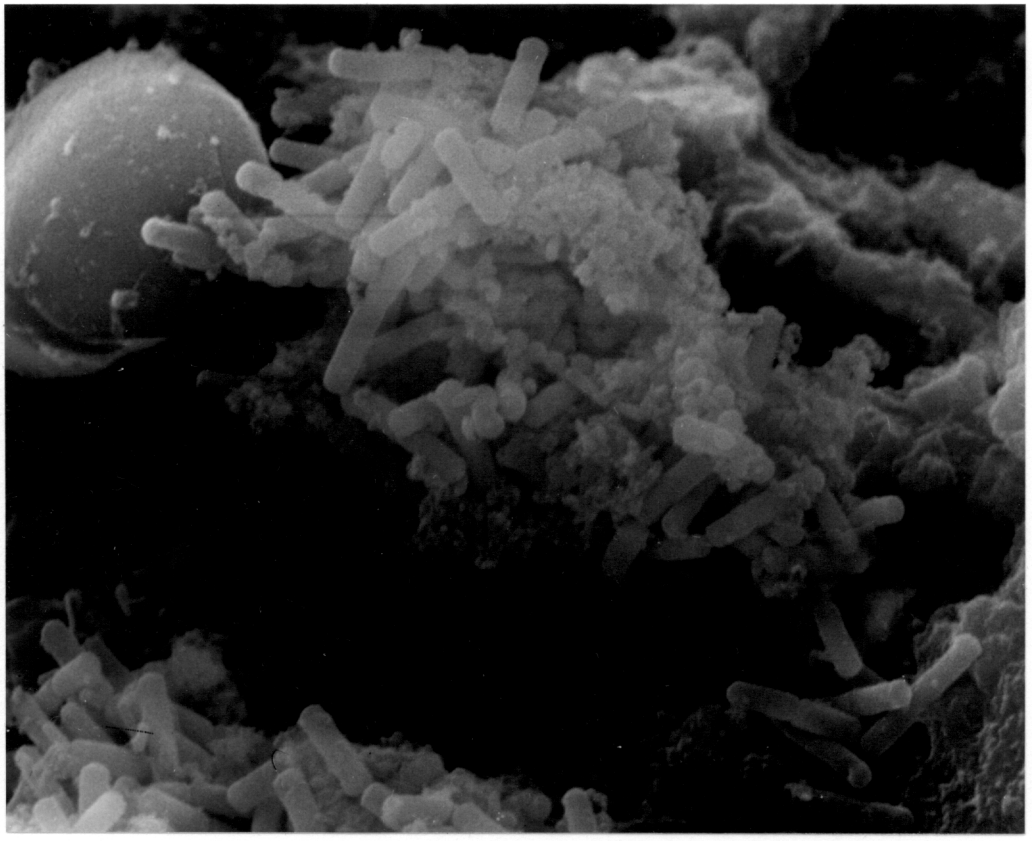

PLATE 24. Scanning electron micrograph of Listeria monocytogenes on buna-N-rubber, 48 hours of exposure. This micrograph is similar to Plate 23. CPD. 3,900 x.

also suggests a sequential process of bacterial attachment to both surfaces studied for this microorganism. The number of bacteria present on both surfaces increased with time as well as the attaching materials.

The concept of "attaching fibrils" is controversial. Frazer and Gilmour (1986) attributed fibril formation to the ethanol dehydration process used for sample preparation in scanning electron microscopy. It is known that bacterial exopolysaccharides were not observed by electron microscopists for some time due to the fact that this molecules do not attract heavy metal stains. The use of ruthenium red (Luft,1971), and alsian blue (Fletcher and Floodgate,1973) enabled the visualization of bacterial glycocalyxes which would be otherwise impossible to see, since the alcohol dehydration process used in electron microscopy for sample preparation almost completely destroys the polysaccharide network which in natural condition is 95-99% water (Characklis,1984; Sutherland,1977). This may explain why bacteria in some of the micrographs obtained appear "bare", without the protective layers of exopolysaccharides, for critical point dried samples. The micrographs of samples prepared by critical point drying showed a striking difference between organisms; each of the three bacteria studied attached to both surfaces at different times, and the interaction of the biological material for each organism with the chemicals used for sample preparation was also different.

For Pseudomonas fragi, cells appeared very "clean" and the attaching fibrils were the main structure visible; however the micrograph of 72 hours (Plate 13) show more material entangled with the attaching fibrils than all the previous ones. Can be speculated that, the amount and chemical composition of the materials produced by bacteria at that time of their growth on the surfaces, allowed those materials to resist better to the dehydration process.

In the case of Bacillus cereus, the protective coating of the biofilm was evident in some of the micrographs (Plates 17, 18, 19, and 20). This suggests that the extracellular polysaccharides produced by this bacterium during the process of attachment to this two surfaces, may be different from that produced by Pseudomonas fragi. Plate 19 shows a freeze dried sample of Bacillus cereus that complements the observations made with critical point dried samples (Plates 14, 15, 16, 17, 18, and 20). The freeze drying process preserves the three dimensional structure reasonably well (Bessis and Weed, 1972), and it may be speculated that when samples are critical point dried, most of the material covering the bacteria is washed away. It is important to use different techniques which complement each other, to obtain a better perspective of what happens in reality. Results obtained with one technique only may lead to false assumptions.

The biofilm of Listeria monocytogenes is different from that of the other two bacteria studied, showing bacteria entangled and diffused through a large mass of material, which is possibly polymeric bacterial exopolysaccharides and other molecules of the biofilm. The chemical composition of the exopolysaccharides produced during the process of attchment by the three bacteria studied, remains to be determined.

The results obtained in this part of the study show the formation of biofilms on food contact surfaces, specifically milk contact surfaces. This fact should be accounted for when cleaning and sanitizing procedures are to be evaluated. It is my opinion that buna-N-rubber may present a bigger challenge to clean and sanitize effectively than teflon, due to its topography Buna-N-rubber has a very irregular surface full of crevices. It is conceivable that when bacteria attach to some of this crevices they become even more difficult to remove than when attached to a

smoother surface.

Another very important factor is time. The micrographs obtained for each of the bacteria studied suggest that the longer bacteria remain on the surface, the harder it will become to remove them from the gasket surfaces. This is probably due to a progressive increase not only of bacterial numbers but also to the more abundant protective biofilm material produced by these bacteria. The protective layers of the biofilm seen in some of the micrographs give an understanding why biofilms are so difficult to eradicate from any system, and why bacteria once incorporated in the biofilm become more resistant to antimicrobials when compared to bacteria tested in suspensions (Costerton et al. (1985); Gristina et al. (1989).

It is conceivable that if bacteria are present in milk or other dairy products which may come into contact with gasket surfaces, they may attach to those surfaces, form a biofilm, resist cleaning and sanitizing. Thus the microorganisms remain in the system and provide a continuous source of contamination for further products that the surface may come into contact with. It can be speculated that cells may be sloughed off from the biofilm and be spread into the surrounding environment (Characklis 1981a) which can be milk or other dairy products, further contaminating other batches of product.

C. Quantitave Analysis of Bacterial Attachment to Teflon and to Buna-N-Rubber Gaskets as Affected by Milk Fat Content

The quantitave study of the attachment of Pseudomonas fragi to buna-N-rubber and to teflon when grown at 21° C and at 4° C was performed and statistical analysis (SAS Institute Inc., SAS Circle, Cary, N.C.) of the results obtained are presented in Tables 1 and 2. It is apparent from these statistical analysis that

TABLE 1
Statistical analysis of the quantitative attachment of Pseudomonas fragi grown at 21° C as effected by milk fat content and by type of gasket material.

Dependant Variable	Source	df	F value
W_1	Gasket	1	44.01*
	Fat	3	3.10*
	Gasket x fat	3	1.66
W_2	Gasket	1	23.55*
	Fat	3	0.54
	Gasket x fat	3	0.46
W_3	Gasket	1	4.75*
	Fat	3	3.32*
	Gasket x fat	3	1.00
Ir	Gasket	1	56.12*
	Fat	3	2.40
	Gasket x fat	3	4.85*
Tt	Gasket	1	44.87*
	Fat	3	0.05
	Gasket x fat	3	0.90

*Significant, with p< or =0.05

W_1--Number of bacteria removed with the first wash.

W_2--Number of bacteria removed with the second wash.

W_3--Number of bacteria removed with the third wash.

Ir--Number of bacteria irreversibly attached to the surface.

Tt--Total number of bacteria attached to the surface (reversibly and irreversibly attached).

TABLE 2
Statistical analysis of the quantitative attachment of Pseudomonas fragi grown at 4° C as effected by milk fat content and by type of gasket material.

Dependant Variable	Source	df	F value
W_1	Gasket	1	13.83*
	Fat	3	2.99*
	Gasket x fat	3	0.10
W_2	Gasket	1	1.76
	Fat	3	3.36 *
	Gasket x fat	3	0.26
W_3	Gasket	1	1.01
	Fat	3	2.26
	Gasket x fat	3	0.57
Ir	Gasket	1	3.09
	Fat	3	0.92
	Gasket x Fat	3	0.80
Tt	Gasket	1	14.36*
	Fat	3	4.65*
	Gasket x Fat	3	0.96

*Significant with p< or =0.05

W_1--Number of bacteria removed with the first wash.

W_2--Number of bacteria removed with the second wash.

W_3--Number of bacteria removed with the third wash.

Ir--Number of bacteria irreversibly attached to the surface.

Tt--Total number of bacteria attached to the surface (reversibly and irreversibly attached).

milk fat content does not seem to play a significant role in the process of attachment of this bacterium to the two surfaces studied; however, the type of gasket material had a significant role. Since milk fat content was not significant in the process of Pseudomonas fragi attachment to the two surfaces studied, the results obtained from all experiments performed at respectively 21° C and 4° C were averaged, and final results are documented in Tables 3 and 4.

Pseudomonas fragi attached in significantly higher numbers to buna-N-rubber than to teflon both at 21° C and at 4° C. When the growth temperature was 21° C (Table 3), at least twice as many bacteria attached to buna-N-rubber than to teflon, and the number of bacteria irreversibly attached to each surface (Ir) is one log cycle lower than the total number of bacteria (Tt) attached to the same surfaces. The difference between those two numbers indicates the number of bacteria reversibly attached to each of the two surfaces and removed with the three wahings. When Pseudomonas fragi was grown at 4° C (Table 4) bacteria attached in lower numbers to both surfaces than when bacteria were grown at 21° C, two log cycles less. The number of irreversibly attached bacteria (Ir) was one log cycle lower than the total (Tt) number of bacteria attached to each one of the surfaces, the difference between the two numbers represents the number of bacteria reversibly attached to each one of the surfaces and removed with the three washings.

Notermans and Kampelmacher (1974) also reported higher bacterial attachment at 21° C, while Butler et al. (1979) found temperature to have very little effect on the extent of bacterial attachment. Stone and Zotolla (1986) reported that Pseudomonas fragi attached to stainless steel at 21° C and at 4° C, while Herald and Zotolla (1988a) found Yersinia enterocolitica to attach better at 21°C than at 35°C and 4°C. Herald and Zotolla (1988b) also reported that Listeria monocytogenes

TABLE 3
Numbers of Pseudomonas fragi attached to buna-N-rubber and teflon, after 12 hours exposure at 21° C.

	Rubber *	Teflon
W_1	3.2×10^4	1.6×10^4
W_2	5.0×10^3	2.6×10^3
W_3	3.2×10^2	1.2×10^2
Ir	6.5×10^3	1.9×10^3
Tt	5.5×10^4	2.1×10^4

* significantly different with $p < \text{or} = 0.05$

W_1--Number of bacteria removed with the first wash, counts/mm^2.

W_2--Number of bacteria removed with the second wash, counts/mm^2.

W_3--Number of bacteria removed with the third wash, counts/mm^2.

Ir--Number of bacteria irreversibly attached to the surface, counts/mm^2.

Tt--Total number of bacteria attached to the surface, (reversibly and irrversibly attached), counts/mm^2.

TABLE 4
Numbers of Pseudomonas fragi attached to buna-N-rubber and teflon, after 12 hours exposure at 4° C.

	Rubber *	Teflon
W_1	6.2×10^2	2.9×10^2
W_2	4.0×10^1	3.0×10^1
W_3	2.0×10^0	4.0×10^0
Ir	6.2×10^1	3.2×10^1
Tt	7.8×10^2	3.8×10^2

* significantly different with $p < \text{or} = 0.05$

W_1--Number of bacteria removed with the first wash, counts/mm^2.

W_2--Number of bacteria removed with the second wash, counts/mm^2.

W_3--Number of bacteria removed with the third wash, counts/mm^2.

Ir--Number of bacteria irreversibly attached to the surface, counts/mm^2.

Tt--Total number of bacteria attached to the surface (reversibly and irreversibly attached), counts/mm^2.

attached to stainless steel at 35°C, 21°C and at10^{0}C, and Mafu et al. (1990) found Listeria monocytogenes to attach to various surfaces both at 4° C and 20^{0}C.

D. Quantitave Analysis of the Affect of Nutrients on the Process of Bacterial Attachment

Statistical analysis of the quantitative attachment of Pseudomonas fragi grown at 21° C to teflon and to buna-N-rubber, as effected by nutrients is documented in Tables 5 to 12. Overall, bacteria attached always in significantly higher numbers to buna-N-rubber than to teflon, the nutrient concentration had a significant role in the process of attachment, and the interaction between the gasket material and the nutrient content was also significant.

When the source of nutrients was skim milk at different concentrations (1:1, 1:4, 1:32), Tables 5 and 6, bacteria attached in significantly higher numbers to buna-N-rubber when the nutrient concentration was minimal. For teflon the highest number of bacteria attached when skim milk was diluted 1:4. When skim milk was diluted 1:32, the number of Pseudomonas fragi reversibly attached and removed with the three washings was comparatively lower than the number of bacteria reversibly attached to both surfaces when the nutrient concentration was higher (undiluted skim milk and diluted 1:4). This was observed for both gasket materials. This fact may suggest a more efficient process of bacterial attachment under less favorable conditions (Costerton, 1981a, b; Costerton et al.,1985). It remains to be determined if bacterial exopolysaccharides synthesized under stress conditions are different from those produced when environmental conditions are favorable.

Tables 7 and 8 present the statistical analysis of the quantitative attach-

TABLE 5
Statistical analysis of the quantitative attachment of Pseudomonas fragi as effected by content of nutrients (different dilutions of skim milk), and by type of gasket material, after 12 hours exposure at 21° C.

Dependant Variable	Source	df	F value
W_1	Gasket	1	71.73*
	Nutrients	2	0.33
	Gask. x Nut.	2	4.19*
W_2	Gasket	1	68.65*
	Nutrients	2	1.39
	Gask. x Nut.	2	3.21
W_3	Gasket	1	48.80*
	Nutrients	2	4.41*
	Gask. x Nut.	2	2.30
Ir	Gasket	1	56.21*
	Nutrients	2	2.01
	Gask. x Nut.	2	4.80
Tt	Gasket	1	166.19*
	Nutrients	2	16.90*
	Gask. x Nut.	2	14.84*

* Significant with $p< or =0.05$

W_1--Number of bacteria removed with the first wash.

W_2--Number of bacteria removed with the second wash.

W_3--Number of bacteria removed with the third wash.

Ir--Number of bacteria irreversibly attached to the surface.

Tt--Total number of bacteria attached to the surface (reversibly and irreversibly attached).

TABLE 6
Numbers of Pseudomonas fragi attached to buna-N-rubber and to teflon as effected by varying concentrations of skim milk and type of gasket material after 12 hours exposure at 21° C

		Dilutions of skim milk 1:1	1:4	1:32
W_1	R	9.90×10^3 A	4.97×10^4 B	1.50×10^4 B
	T	4.20×10^3 A	9.50×10^3 A	8.30×10^2 A
W^2	R	1.40×10^3 A	8.20×10^3 B	6.10×10^3 B
	T	3.30×10^1 A	1.30×10^2 A	7.20×10^1 A
W_3	R	4.50×10^2 A	2.90×10^3 B	4.20×10^2 B
	T	5.00×10^1 A	6.00×10^1 A	9.00×10^0 A
Ir	R	2.14×10^3 A	9.70×10^3 B	2.87×10^4 C
	T	6.00×10^2 A	2.30×10^3 B	8.20×10^2 A
Tt	R	1.33×10^4 A	8.17×10^4 B	6.95×10^4 B
	T	5.90×10^3 B	1.30×10^4 C	2.30×10^3 A

Averages in the same row with different letters are significantly different ($p < or = 0.05$).

W_1--Number of bacteria/mm^2 removed with the first wash.

W_2--Number of bacteria/mm^2 removed with the second wash.

W_3--Number of bacteria/mm^2 removed with the third wash.

Ir--Number of bacteria/mm^2 irreversibly attached to the surface.

Tt--Total number of bacteria/mm^2 attached to the surface (reversibly and irreversibly attached).

R --Buna-N-rubber.

T --Teflon.

ment of Pseudomonas fragi to buna-N-rubber and to teflon when the nutrient source was casein in various dilutions. The greatest number of bacteria attached to both surfaces when the nutrient concentration was maximal (undiluted casein). For buna-N-rubber the number of bacteria attached when the nutrient concentration was minimal was not significantly different from that observed with maximal nutrient content. However, when the dilution of nutrients was 1:4 the number of bacteria attached to buna-N-rubber was significantly lower. The greatest number of Pseudomonas fragi attached to teflon when the nutrient concentration was maximal, and, inversely, the lowest number of bacteria attached to teflon when the nutrient concentration was maximal. Regardless of the concentration of nutrients, the number of bacteria irreversibly attached to both surfaces was roughly one half of the total number of bacteria attached (Table 8).

Results for experiments on the attachment of Pseudomonas fragi grown in casein and lactose is given in Tables 9 and 10. The greatest number of bacteria attached to buna-N-rubber when the nutrient concentration was maximal; however, this number was not significantly different from the numbers of bacteria attached to the same surface at the other two nutrient concentrations (Table 10) with averages belonging to the same group. In the case of teflon, bacteria attached in significantly greater numbers when the concentration of nutrients was maximal (whole casein with lactose), followed by the 1:4 and 1:32 dilutions.

Tables 11 and 12 present the statistical analysis for the quantitative attachment of Pseudomonas fragi to both surfaces when whey with lactose was used as a source of nutrients. In the case of buna-N-rubber bacteria attached in higher numbers when nutrients were maximal; however, when the nutrient concentration was minimal (1:32) the number of bacteria attached to buna-N-rubber was not

TABLE 7
Statistical analysis of the quantitative attachment of Pseudomonas fragi as effected by content of nutrients (different dilutions of casein), and by type of gasket material, after 12 hours exposure at 21° C.

Dependant Variable	Source	df	F value
W_1	Gasket	1	117.56*
	Nutrients	2	39.96*
	Gask. x Nut.	2	3.83*
W_2	Gasket	1	43.48*
	Nutrients	2	25.39
	Gask. x Nut.	2	10.30
W_3	Gasket	1	74.71*
	Nutrients	2	23.71*
	Gask. x Nut.	2	3.49*
Ir	Gasket	1	113.69*
	Nutrients	2	19.23*
	Gask. x Nut.	2	21.99*
Tt	Gasket	1	115.64*
	Nutrients	2	15.20*
	Gask. x Nut.	2	13.98*

* Significant with $p \leq 0.05$

W_1--Number of bacteria removed with the first wash.

W_2--Number of bacteria removed with the second wash.

W_3--Number of bacteria removed with the third wash.

Ir--Number of bacteria irreversibly attached to the surface.

Tt--Total number of bacteria attached to the surface (reversibly and irreversibly attached).

TABLE 8
Numbers of Pseudomonas fragi attached to buna-N-rubber and to teflon as effected by varying concentrations of casein and type of gasket material after 12 hours exposure at 21° C

		Dilutions of casein		
		1:1	1:4	1:32
W_1	R	1.20×10^4 B	5.70×10^3 A	9.80×10^3 A
	T	7.70×10^3 C	9.90×10^2 B	7.50×10^2 A
W_2	R	8.70×10^3 B	4.80×10^2 A	2.50×10^2 A
	T	2.40×10^2 C	4.40×10^1 B	2.10×10^1 A
W_3	R	2.60×10^2 C	4.50×10^1 A	1.10×10^2 B
	T	8.80×10^2 B	1.50×10^1 A	1.10×10^1 A
Ir	R	8.90×10^3 B	4.30×10^3 A	1.20×10^4 B
	T	4.30×10^2 C	7.38×10^2 B	3.24×10^2 A
Tt	R	2.30×10^4 B	1.10×10^4 A	2.20×10^4 B
	T	1.40×10^4 B	2.37×10^3 A	1.50×10^3 A

Averages in the same row with different letters are significantly different, ($p < or = 0.05$)

W_1--Number of bacteria/mm^2 removed with the first wash.

W_2--Number of bacteria/mm^2 removed with the second wash.

W_3--Number of bacteria/mm^2 removed with the third wash.

Ir--Number of bacteria/mm^2 irreversibly attached to the surface.

Tt--Total number of bacteria/mm^2 attached to the surface (reversibly and irreversibly attached).

R --Buna-N-rubber.

T --Teflon.

TABLE 9
Statistical analysis of the quantitative attachment of Pseudomonas fragi as effected by content of nutrients (different dilutions of casein with lactose), and by type of gasket material after 12 hours exposure at 21°C.

Dependant Variable	Source	df	F value
W_1	Gasket	1	72.67*
	Nutrients	2	2.41
	Gask. x Nut.	2	3.70*
W_2	Gasket	1	45.68*
	Nutrients	2	4.37*
	Gask. x Nut.	2	2.74
W_3	Gasket	1	50.75*
	Nutrients	2	0.07*
	Gask. x Nut.	2	5.41*
Ir	Gasket	1	128.50*
	Nutrients	2	5.18*
	Gask. x Nut.	2	13.20*
Tt	Gasket	1	223.74*
	Nutrients	2	15.47*
	Gask. x Nut.	2	8.22*

*Significant with $p \leq 0.05$

W_1--Number of bacteria removed with the first wash.

W_2--Number of bacteria removed with the second wash.

W_3--Number of bacteria removed with the third wash.

Ir--Number of bacteria irreversibly attached to the surface.

Tt--Total number of bacteria attached to the surface (reversibly and irreversibly attached).

TABLE 10
Numbers of Pseudomonas fragi attached to buna-N-rubber and to teflon as effected by varying concentrations of casein with lactose and type of gasket material after 12 hours exposure at 21° C

		Dilutions of casein with lactose 1:1	1:4	1:32
W_1	R	1.10×10^4 B	9.80×10^3 B	2.70×10^3 A
	T	1.80×10^2 A	3.00×10^2 A	2.90×10^2 A
W_2	R	2.00×10^3 C	8.70×10^2 B	4.00×10^2 A
	T	2.00×10^1 A	9.00×10^0 A	1.50×10^1 A
W_3	R	1.80×10^2 A	1.00×10^2 A	1.00×10^2 A
	T	1.00×10^1 A	7.00×10^0 A	3.00×10^0 A
Ir	R	6.30×10^3 A	4.90×10^3 A	8.70×10^3 A
	T	5.50×10^3 C	9.70×10^2 B	4.20×10^2 A
Tt	R	2.30×10^4 A	1.80×10^4 A	1.87×10^4 A
	T	7.20×10^3 C	2.10×10^3 B	1.10×10^3 A

Averages in the same row with different letters are significantly different, ($p < or = 0.05$)

W_1--Number of bacteria/mm^2 removed with the first wash.

W_2--Number of bacteria/mm^2 removed with the second wash.

W_3--Number of bacteria/mm^2 removed with the third wash.

Ir--Number of bacteria/mm^2 irreversibly attached to the surface.

Tt--Total number of bacteria/mm^2 attached to the surface (reversibly and irreversibly attached).

R --Buna-N-rubber.

T --Teflon.

TABLE 11

Statistical analysis of the quantitative attachment of Pseudomonas fragi as effected by content of nutrients (different dilutions of whey with constant levels of lactose), and by type of gasket material after 12 hours exposure at 21°C.

Dependant Variable	Source	df	F value
W_1	Gasket	1	61.33*
	Nutrients	2	10.16
	Gask. x Nut.	2	0.07
W_2	Gasket	1	155.99*
	Nutrients	2	38.25*
	Gask. x Nut.	2	6.89
W_3	Gasket	1	86.14*
	Nutrients	2	27.33*
	Gask. x Nut.	2	14.93*
Ir	Gasket	1	106.48*
	Nutrients	2	62.91*
	Gask. x Nut.	2	12.81*
Tt	Gasket	1	150.01*
	Nutrients	2	63.80*
	Gask. x Nut.	2	9.49*

*Significant with $p \leq 0.05$

W_1--Number of bacteria removed with the first wash.

W_2--Number of bacteria removed with the second wash.

W_3--Number of bacteria removed with the third wash.

Ir--Number of bacteria irreversibly attached to the surface.

Tt--Total number of bacteria attached to the surface (reversibly and irreversibly attached).

TABLE 12
Numbers of Pseudomonas fragi attached to buna-N-rubber and to teflon as effected by varying concentrations of whey with constant levels of lactose and by type of gasket material after 12 hours exposure at 21° C.

		Dilutions of whey with lactose		
		1:1	1:4	1:32
W_1	R	4.30×10^4 B	5.30×10^3 A	8.60×10^3 A
	T	9.60×10^3 B	9.70×10^2 A	5.70×10^2 A
W_2	R	1.20×10^3 C	1.80×10^2 A	5.50×10^2 B
	T	2.80×10^2 B	2.70×10^1 A	2.20×10^1 A
W_3	R	1.40×10^2 B	4.00×10^1 A	2.00×10^2 B
	T	1.20×10^2 B	7.00×10^0 A	9.00×10^0 A
Ir	R	1.10×10^4 B	5.67×10^3 A	2.00×10^4 B
	T	8.03×10^3 C	9.30×10^2 A	1.20×10^3 B
Tt	R	6.10×10^4 B	1.04×10^4 A	3.16×10^4 B
	T	2.10×10^4 B	2.20×10^3 A	2.12×10^3 A

Averages in the same row with different letters are significantly different, ($p < or = 0.05$)

W_1--Number of bacteria/mm^2 removed with the first wash.

W_2--Number of bacteria/mm^2 removed with the second wash.

W_3--Number of bacteria/mm^2 removed with the third wash.

Ir--Number of bacteria/mm^2 irreversibly attached to the surface.

Tt--Total number of bacteria/mm^2 attached to the surface (reversibly and irreversibly attached).

R --Buna-N-rubber.

T --Teflon.

significantly different. The number of bacteria irreversibly attached to the same surface was higher (in comparison to the reversibly attached) when the nutrient concentration was minimal, suggesting a more efficient process of bacterial attachment when nutrients were less available.

Bacteria attached to teflon in higher numbers when nutrient concentration was maximal and vice-versa. For most cases at least half of the bacteria were removed with the three washings (reversibly attached bacteria).

Pseudomonas fragi attached always in significantly higher numbers to buna-N-rubber than to teflon. Buna-N-rubber is a more hydrophobic surface than teflon. Dexter (1979) and Dexter et al. found minimal bacteria attachment to hydrophobic substrata and maximal attachment to hydrophillic surfaces, while others (Carson and Allsopp, 1980; Loeb, 1977; Fletcher and Loeb, 1979. 1976; Pringle and Fletcher,1986), reported opposite findings. Our results agree with those of the latter groups.

The variations found in bacterial attachment as affected by nutrients in different concentrations has also been reported by Marshall et al. (1971a) who found the attachment of a marine pseudomonad to be dependent on the concentration of glucose used, with attachment favored at lower concentrations of glucose. In our study results varied. However, a trend was observed with teflon, with Pseudomonas fragi attaching mostly in higher numbers when nutrient concentration was maximal and vice-versa. With buna-N-rubber the trend, for the most part, was reversed.

VI. Summary and Conclusions

Water contact angle measurements (151.1° for buna-N-rubber versus 112° for teflon) show that buna-N-rubber has a more hydrophobic surface than teflon, has less surface free eneryge than teflon. Electron microscopic studies were used to characterize the topography of both gasket materials: buna-N-rubber had a very irregular and rough surface full of crevices, while teflon was mostly a smooth surface with random rough spots. X-ray micro-analysis was used to analyze the elemental chemical composition of both materials: buna-N-rubber contained sulfur, silicone and calcium, while teflon only contained fluorine.

The very irregular topograpgy and less surface free energy of buna N-rubber may account for the fact that this surface consistently attached Pseudomonas fragi in higher numbers than teflon. The role of the elemental composition of buna-N-rubber (presence of Ca^{++}) in the process of bacterial attachment needs further investigation, possibly using electron spectroscopy (ESCA).

All three bacteria studied, Pseudomonas fragi, Bacillus cereus and Listeria monocytogenes, each had the ability to irreversibly attach to both teflon and buna-N-rubber under the conditions studied, with Pseudomonas fragi attaching at 2 hours of bacterial growth, Bacillus cereus at 12 hours and Listeria monocytogenes at 24 hours. The number of bacteria and fibrils present on both surfaces increased with time. Biofilms of each of the three bacteria studied are very different. The protective layer of each biofilm resisted the alcohol dehydration process in a unique way. This may suggest that slime materials synthesized by each bacteria are different. The chemical composition of these materials needs to be further investigated.

The affect of milk fat content and temperature in the process of Pseudomo-

nas fragi attachment was examined. Milk fat content did not play a significant role; however, this bacterium attached in significantly higher numbers to buna-N-rubber than to teflon. At 21°C this bacterium attached to both surfaces in higher numbers than at 4°C. This is probable due to the fact that at 4° C bacteria grow at a much slower rate than at 21° C. Larger numbers of bacteria were present on both surface at 21° C due to the faster rate of bacterial growth on the surfaces (once attached) at this temperature, rather than due to just greater bacterial attachment.

When the effect of nutrient concentration (skim milk, casein with and without lactose, and whey with lactose, respectively, at different dilutions)was studied, the nutrient concentration played a significant role: with buna-N-rubber a trend was observed. Significantly higher numbers of bacteria attached when nutrients were minimal. With teflon the observed trend was reversed. However, in all cases, Pseudomonas fragi attached in significantly higher numbers to buna-N-rubber than to teflon.

The very irregular topography and the less surface free energy of buna-N-rubber may explain why Pseudomonas fragi attached in significantly higher numbers to this surface than to teflon. The greater hydrophobicity of buna-N-rubber may allow this surface to attach greater numbers of bacteria. More hydrophillic surfaces become covered with a layer of water molecules, that form a barrier and may exclude bacteria from the surface. The very irregular topography of buna-N-rubber may allow bacteria to become entrapped in the many crevices found on the surface.

The model described allows to count bacteria attached to any surface without killing the bacteria, and represents a new way of counting bacteria attached to a surface. The model is a more realistic way of studying minimum bactericidal

concentrations of bacterial biofilms and may be used in any scientific field.

Gaskets of dairy processing plants can be regarded as a possible source of contamination. Bacterial biofilms can form on gasket surfaces and they may resist cleaning and sanitizing, remain on the surfaces and perpetuate the contamination cycle.

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