EFFECT OF ENVIRONMENTAL STRESS ON THE ABILITY OF *LISTERIA MONOCYTOGENES* SCOTT A TO ATTACH TO FOOD CONTACT SURFACES

by

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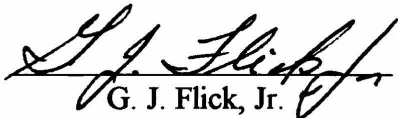

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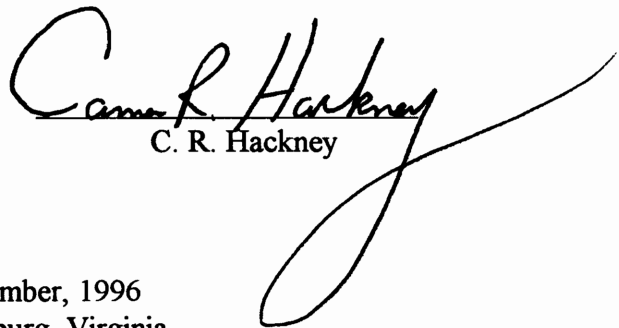

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(ABSTRACT)

The attachment and detachment of *Listeria monocytogenes* Scott A to Buna-N rubber and stainless steel under varying conditions of temperature and pH were investigated. Numbers of attached cells increased with increasing attachment temperatures (10° to 45°C) and time (up to 120 min) for both Buna-N rubber and stainless steel. Cells attached at higher levels on stainless steel at all temperature and pH levels investigated. Rate of attachment was found to be significantly lower at 10°C than 30° and 45°C on Buna-N rubber. When *L. monocytogenes* was grown at 10°, 30°, and 42°C before exposure to Buna-N rubber at 30°C, differences in rates of adhesion were not significant. A downward shift in the cell suspension holding temperature immediately prior to attachment to Buna-N rubber at 10°C resulted in reduced adhered cell populations. A similar upward shift did not affect attachment at 45°C. Altering the pH of the attachment environment within the pH range of 4 to 9 did not affect the maximum levels of attached cells to Buna-N rubber. However, the measured rates of adhesion indicated slower attachment occurs under alkaline conditions. Growth pH was also found to significantly affect rates of attachment

and maximum adhered cell populations to Buna-N rubber. Compared to Buna-N rubber, the rate of attachment to stainless steel was markedly more rapid for all temperature and pH conditions studied and could not be calculated. The ease of removal for cells adhered to Buna-N rubber was significantly affected by growth temperature, but not growth pH. Significant differences in detachment were also found between Buna-N rubber and stainless steel, inferring a stronger attachment to Buna-N rubber. Cell surface hydrophobicity was affected by both growth temperature and growth pH, but differences in hydrophobicity could not be correlated to differences in rates of attachment. Addition of 0.01% trypsin to the attachment medium during cell suspension exposure to both test surfaces resulted in a 99.9% reduction in the adhered cell population when compared to controls. This suggests that proteins may play a role in the initial attachment process for *L. monocytogenes*.

To my parents

who taught me to put one foot in front of the other

and to my husband

whose love and support makes each step more enjoyable.

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I. INTRODUCTION

Over the past decade, *Listeria monocytogenes*, has been regarded as one of the leading causes of food-borne illness. The organism has been found in a wide range of food products including, dairy, meat, poultry, seafood as well as fruits and vegetables (NACMCF, 1991). The infective dose is not known but varies with host susceptibility. Healthy individuals may ingest low levels of the organism unknowingly without occurrence of illness. However, a mortality rate as high as 30-40% has been documented for the immunocompromised portion of the population (Sutherland, 1989). Its high mortality rate, wide occurrence in nature and ability to grow at refrigerated temperatures has prompted some countries to implement a zero tolerance of *L. monocytogenes* in ready-to-eat foods.

L. monocytogenes is ubiquitous in nature, being found on soil, feces, plants and in water samples (NACMCF, 1991). Consequently, it is frequently found on raw materials used in food processing. Cox et al. (1989) demonstrated that listeriae could be found in all types of food production environments. Once present in the processing environment, control of *L. monocytogenes* has proven to be difficult. Refrigerated, moist environments provide conditions which allows survival and growth. Although improvements have been made in controlling *L. monocytogenes* in food systems, its presence in the processing environment is still readily found.

Both physical and chemical treatments are used in food processing to eliminate and control the presence of pathogenic and spoilage microorganisms in foods. When cleaning

and sanitizing regimes are poorly executed or inadequate, microorganisms may remain in the processing environment in an injured state. It has been shown that sublethal treatments or stresses can result in unique adaptive responses. For example, Foster and Hall (1990) reported that when *Salmonella typhimurium* was first preshocked with a mild acid (pH 5.5), cells were 100 to 1,000 times more resistant to a subsequent strong acid challenge than their unadaptive counterparts. Kroll and Pratchett (1992) were also able to demonstrate an acid tolerance response in *Listeria*. This increased resistance to a secondary stress is well documented for a number of stresses such as heat, anaerobiosis, oxidation, starvation and ethanol in a variety of microorganisms (Van Bogelen et al., 1987; Nystrom, 1990; Murano and Pierson, 1992). Increased resistance to treatments which are normally lethal, is due to the production of specific proteins triggered by the presence of sublethal levels of that stress (Foster, 1991). Recent studies indicate that different stresses may trigger the production of similar proteins (Jenkins et al., 1988; Leyer and Johnson, 1993; Lou and Yousef, 1996). In such cases, the microorganism not only exhibits an increased tolerance to lethal levels of that stress which triggered the protein production, but also exhibits an increased tolerance to other stresses which induce the production of similar proteins.

Exposure to stress alters more than the profile of proteins produced (Marshall, 1992). For example, starvation stress not only results in a shift in the production of proteins but is often accompanied by an increase in cell number, a decrease in cell size, an increase in cell surface hydrophobicity and an increase in adhesiveness. It has been

suggested that these factors make the bacteria more prone to adhere to solid surfaces where they benefit from an enhanced nutrient status (Marshall, 1992). Continued adhesion and growth of adhering bacteria eventually leads to biofilm formation. Biofilms present a problem to the food industry, in that, if allowed to persist in the processing environment they can serve as a source of contamination affecting the quality and safety of processed foods.

Microorganisms existing in biofilms have been shown to have an increased resistance to antimicrobials as compared to free-living microorganisms. This increased resistance may result from nutrient limitation in the biofilm (Brown and Gilbert, 1993). Numerous studies have demonstrated that *L. monocytogenes* existing in a biofilm exhibits increased resistance to common sanitizers (Ren and Frank 1993, Ronner and Wong 1993, Lee and Frank 1991, Frank and Koffi 1990).

The initial stage of biofilm formation in the processing environment is the adhesion of microorganisms to food contact surfaces. To date, the majority of biofilm research in the food industry has focused on the ability of microorganism to resist environmental stresses (Hood and Zottola, 1995). Very little work has addressed the effect of these environmental stresses on the ability of microorganisms to attach to different surfaces. For those microorganisms in which a zero tolerance is in effect, attached bacteria may be as significant a threat to the production of safe food as are well developed biofilms. A better understanding of the environmental conditions which favor attachment should aid in the prevention of adhered bacteria and subsequent biofilm formation. Therefore the objective of this study is to determine in a quantitative manner the effects of environmental

stresses on the ability and rate of attachment *Listeria monocytogenes* Scott A to food contact surfaces.

II. REVIEW OF LITERATURE

A. *Listeria monocytogenes*

1. Characteristics of the organism

Listeria monocytogenes is a gram-positive, non-sporeforming, rod 0.4-0.5 μm in diameter and 0.5-2 μm in length. The organism is a facultative anaerobe but prefers microaerophilic conditions with 5-10% CO_2. It is catalase positive, oxidase negative and β-hemolytic. Colonies are low convex shaped, ≤ 1.5 mm, with a characteristic bluish appearance when observed with obliquely transmitted light. The organism possesses peritrichous flagella which give it a characteristic tumbling motion. Motility is temperature dependent, with 20°C being the optimum temperature to demonstrate motility. *Listeria monocytogenes* has a wide temperature range for growth between 1° and 45°C and an optimum growth temperature between 30°- 37°C. The growth pH range is between 4.1 to 9.6. Growth occurs up to 10% NaCl, however, *L. monocytogenes* is able to survive in up to 25.5% NaCl at 4°C. An extensive review of the growth characteristics of *L. monocytogenes* has been provided by Ryser and Marth (1991).

There are at least 13 serotypes of *L. monocytogenes* as determined by the somatic antigens (1-4) and the flagellar or H antigens (a-e). Three of these serotypes, 4b, 1/2b, and 1/2a, account for 90% of all human listeriosis,. Of these serotypes, 4b and 1/2a have been responsible for the majority (59 and 18%, respectively) of foodborne disease outbreaks (Ryser and Marth, 1991).

All strains of *L. monocytogenes* produce listeriolysin O (LLO) an essential virulence factor that is responsible for β-hemolysis of erythrocytes and the destruction of phagocytic cells. Most, but not all, strains that produce LLO are β-hemolytic. The pathogenesis has been divided into four stages: internalization, escape from a vacuole, nucleation of actin filaments and cell-to-cell spread (Portnoy et al., 1988). LLO aids in the survival of *L. monocytogenes* once engulfed in the phagocyte. Enzymes produced by *L. monocytogenes,* such as catalase (CA) and superoxide dismutase (SOD), are thought to enhance survival inside the phagocytic vacuole by detoxifying cytotoxic oxidants. Although these enzymes may contribute to virulence, they are not considered essential for pathogenicity. LLO is an extracellular protein with a molecular weight of 60,000 daltons, consisting of 504 amino acids. Optimum production of LLO occurs during exponential growth, with maximum levels occurring after 8 to 10 h of growth (Geoffroy et al., 1989).

2. Clinical features of listeriosis

Illness caused by *Listeria monocytogenes* is most likely to occur in immunocompromised individuals, pregnant women, fetuses and young children, and the elderly. Though not as common, *L. monocytogenes* also causes disease in otherwise healthy persons with no recognized predisposing factors. Compared to the overall incidence of listeriosis, 0.8 per 100,000 , the incidence for pregnant women, persons over 70 years of age, and persons with acquired immune deficiency syndrome have been estimated to be 12.4 per 100,000 live births, 2.1 per 100,000 population, and 200 per 100,000 population, respectively (NACMCF, 1991). The severity of the illness depends,

to a large extent, upon the host factors. Symptoms of the illness range from flu-like symptoms and mild diarrhea to septicemia, meningitis and in pregnant women, stillbirth or miscarriages. Listeriosis is treated with antibiotics such as ampicillin, penicillin or erythromycin, however, there is still a high mortality rate. Of the reported cases, 25% end in death as a result of the infection (NACMCF, 1991).

3. Epidemiology

Due to the outbreaks of listeriosis in the early 1980s, the Centers for Disease Control and Prevention (CDC) undertook an active surveillance project in to define the epidemiological characteristics of listeriosis. For the U.S., majority of cases of listeriosis are sporadic. Surveillance data indicate that 2000 cases and 450 deaths occur each year in the United States (Schuchat et al., 1992). A higher incidence may be more realistic due to lack of reporting as has been typical of foodborne illnesses.

Epidemiological evidence indicates that exposure to *L. monocytogenes* is mostly foodborne. (Broome et al., 1990). Outbreaks occurring in the 1980s in North America were associated with cole slaw, pasteurized milk, and Mexican-style cheese. The CDC conducted a case-control study, as part of their active surveillance program, to identify foods commonly associated with cases of listeriosis. Twenty percent of all cases reported could be attributed to undercooked poultry and hotdogs that had not been reheated by the consumer. Persons who had eaten soft cheese were found to have 2.6 times the risk of listeriosis and persons who had eaten foods from a delicatessen counters were found to

have 1.6 times the risk of listeriosis when the effects of other foods were controlled. The study also reported an association of undercooked poultry and sporadic listeriosis, but only within the immunocompromised population (Schuchat et al., 1992).

The infective dose of a food-borne pathogen depends on host conditions, virulence and infectivity of the pathogen, type and amount of the food consumed, as well as the level of the pathogen in the food. In a few cases, the numbers of listeriae present in those foods implicated in a particular food-borne illness have been reported. In the outbreak caused by Mexican-style cheese, samples contained 10^3 to 10^4 *L. monocytogenes* per g of cheese (Linnan, et al., 1988). In another case involving an immunocompromised individual, the incriminated food sample contained > 1100 *L. monocytogenes* per g of turkey franks (Wenger et al., 1990). Due to the time between onset of symptoms and laboratory analysis and the fact that *L. monocytogenes* can grow at refrigeration temperatures, the levels of listeriae in the incriminated foods should be carefully considered. As the general age of the population increases and a larger segment of the population is living with immunosuppressive illnesses, the percent of the population at risk for listeriosis will also increase.

4. Distribution

L. monocytogenes is ubiquitous in nature and is able to survive in a wide range of environmental conditions. The organism has been isolated from soil, sewage, animal feces and decaying vegetation including silage and other animal feeds (Gray, 1963). *L.*

monocytogenes was found in all 50 samples of sewage, sewage sludge and river water examined in the United Kingdom, often in excess of *Salmonella* (Watkins and Sleath, 1981). *Listeria* has also been found in large numbers in mud samples indicating that this organism is able, not only to survive, but to multiply, particularly in moist environments (Weis and Seeliger, 1975).

Prior to 1980, listeriosis was primarily a veterinary concern, occurring in a wide variety of animals both domestic and wild. *L. monocytogenes* has been isolated from at least 42 species of mammals and 22 species of birds, as well as fish and crustaceans (NACMCF, 1991). Many animals appear to be healthy carriers of *L. monocytogenes*. The organism has been isolated from a number of animals that either died from natural causes or were slaughtered for human consumption.

5. Association with foods

The incidence of *L. monocytogenes* in selected food products has been reported as high as 45% in milk, 95% in pork, 60% in raw poultry, 79% in ground beef, and 30% in some vegetables (Jay, 1992). The Food and Drug Administration (FDA) reported the incidence of *L. monocytogenes* to be 5.1% in shrimp, 4.9% in cooked lamb, 1.4% in cheddar cheese, and 3.8% in novelty ice cream (Doyle, 1989). Green (1990) found 7.1% of raw beef samples, 19.3% of raw broiler necks and backs and 2.8% of ready-to-eat meats from 4,105 processing plants to the United States were positive for *L. monocytogenes*. A survey of ready-to-eat seafood, conducted as part of the EC

Coordinated Food Control Program (ECCFCP), revealed the incidence of *L. monocytogenes* to be 4.1% in smoked fish and 11% in fish and shellfish. *Listeria* has also been isolated from shrimp, crabmeat (Archer, 1988), shellfish and fish (Brackett, 1988). Recent surveys conducted in the United Kingdom in ready-to-eat foods indicated that *L. monocytogenes* is generally present at levels < 1000 CFU/g (Anonymous, 1996). The ubiquitous nature of *L. monocytogenes* allows easy access to food products during various phases of production, processing, manufacture and distribution. Genigeorgis et al. (1990) showed that during processing, chilling, packaging and final retailing of processed turkey, the percentage of samples containing *Listeria* spp. increased from 4% to 23%. The prevention of listeriae in raw foods is difficult to achieve. Its presence in processed and packaged foods which have been treated with a listericidal process would suggest post-processing contamination from the environment has occurred and that strict control measures must be implemented (Sutherland, 1989).

6. Persistence in food processing environments and foods

In an environmental study of the prevalence of *Listeria* spp., Cox et al. (1989) found *Listeria* spp. in all food plants studied (frozen food factories, dairy plants, Italian cheese plants, ice-cream production, potato processing) except in dry culinary food factories. *L. monocytogenes* comprised 5-20% of the listeriae isolated from the different factories. *Listeriae* was most often found in drains (54%), followed by stagnant water (46%), floors (45%), residues (33%), and processing equipment (19%). Since *L.*

monocytogenes can grow over such a wide temperature and pH range, the organism may be expected to survive for long periods of time in food processing environments. In a study conducted by Stanfield et al. (1987), viable *L. monocytogenes* was recovered from the surface of inoculated plastic and waxed cardboard milk containers after 14 days storage. The extent to which processed foods become contaminated with *L. monocytogenes* depends on the degree of contact with contaminated surfaces. It has been suggested that microorganisms present in biofilms contribute to post-processing contamination (Mafu et al., 1990; Hood and Zottola, 1995).

The landmark outbreak of listeriosis in 1985, in which Mexican-style cheese was implicated, led to numerous studies investigating the growth and survival of *L. monocytogenes* in dairy products. *L. monocytogenes* has been shown to persist for extended periods in cottage cheese during storage at 3°C (Ryser et al., 1985). *L. monocytogenes* was able to grow to 10^6-10^7 CFU/g during ripening of Camembert cheese inoculated with levels of 10^4-10^5 CFU/g (Ryser and Marth, 1987) . Other examples of foods inoculated with *L. monocytogenes* in which the organism was able to either survive or grow include ready-to-eat processed meats, Finnish sausage, ground beef, ravioli, and lettuce. An extensive review of the growth and survival of *L. monocytogenes* in foods is provided by Ryser and Marth (1991) and more recently by ICMSF (1996). These reviews also provide excellent summaries of the vast studies performed since the early 1980s to investigate the organism's ability to survive various preservative strategies including combinations of heat, salt, pH (acids), antimicrobials, and gas atmospheres. These studies

indicate that the overall persistence of *L. monocytogenes* in foods is consistent with their survival in many nonfood environments (Jay, 1992).

B. Environmental Stress

1. Increased resistance/tolerance

Both physical and chemical treatments are used in the food processing environment to eliminate and control the presence of pathogenic and spoilage bacteria in foods. Sublethal treatments may result in injured cells which are able to repair and proliferate once placed in an unstressed environment. Bacterial injury, as it relates to the control of microorganisms in food, has primarily been studied with treatments of freezing, heating, irradiation, and antimicrobials. Historically, injured cells have been defined as those cells which can form colonies on nonselective plating media but not on selective plating media (Ray, 1986). From this definition, it would follow that cells exposed to sublethal treatments (stresses) are more susceptible to adverse environments or secondary stresses to which non-injured cells can readily adapt.

There is now evidence that when cells are first exposed to a sublethal stress, they can acquire increased resistance to subsequent lethal levels of that same stress. For example, Foster and Hall (1990) reported that when *Salmonella typhimurium* was first preshocked with a mild acid (pH 5.8), cells were 100 to 1,000 times more resistant to a subsequent strong acid challenge than their unadapted counterparts. Kroll and Patchett (1992) were also able to demonstrate acid tolerance response in *Listeria*. This increased

resistance to a secondary stress is not limited to acid tolerance. *Listeria, Salmonella*, and *Escherichia coli* O157:H7 have all been shown to have an increased heat resistance after being heat shocked at elevated temperatures (Bunning et al., 1990; Christman et al., 1985; Murano and Pierson, 1992). A common requirement of increased resistance/tolerance to secondary stresses is protein synthesis (Jenkins et al., 1988; Foster, 1991).

2. Cross protection

Procaryotic cells are known to respond to environmental or chemical stress by inducing specific sets of genes termed stimulons. Each stimulon, and the proteins it produces, is characteristic for a specific stress. Those proteins produced as a consequence of exposure to a sublethal stress confer protection to a subsequent lethal exposure of that same stress. Examples of well characterized stimulons include those for heat shock and oxidative stress (VanBogelen et al., 1987). Recent studies have indicated that stimulons may regulate the production of similar proteins. Two stimulons which regulate the production of common proteins can produce a cross protective effect for the two environmental stresses inducing these stimulons. Therefore, not only do the cells have an increased resistance to the stress triggering that stimulon, but to any other stress which triggers the production of similar proteins. This cross protective effect was first reported by Jenkins et al., (1988), when glucose or nitrogen starved cultures of *E. coli* exhibited enhanced resistance to heat and hydrogen peroxide challenge. Again, protein synthesis was essential for these two cross protections since the addition of chloramphenicol, an

inhibitor of protein synthesis, prevented the development of thermal and oxidative resistance. Leyer and Johnson (1993) reported cross protection to heat, salt, lactoperoxidase system, and surface active agents (crystal violet, and polymixin B) when *S. typhimurium* was first adapted to acid. They indicated that in addition to altered protein synthesis, changes were also noted in the cell surface properties brought about by classical constitutive pH homeostasis.

In the environment, microorganisms may endure more than one stress and therefore exhibit complex environmental stress responses. However, to date, there has been few reports on the combined effects of multiple environmental stresses. This area was touched upon when Nystrom (1990) reported on the response of multiple nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. When cells were starved for glucose, amino acids, ammonium and phosphate simultaneously, a total of 66 proteins were induced. They found that the total number of proteins produced by multiple starvation significantly exceeded the sum of proteins induced by starvation for each of the individual nutrients. In addition, 13 of those proteins induced by multiple starvation were unique and not observed when cells were starved for any one of the individual nutrients. Pre-starvation for 1 h was shown to enhance survival of subsequent multiple-nutrient starvation. When other marine *Vibrio* spp. were subjected to a severe lack of energy and nutrients, cells demonstrated an increased resistance to autolysis, sonic lysis, hydrostatic pressure, heat and cold shock and increased adherence to a variety of surfaces (Nystrom et

al., 1990). It is possible that combinations of different environmental stresses may produce responses unique from those obtained from any one individual environmental stress.

3. Effect on virulence of *Listeria monocytogenes*

Microorganisms respond to stresses, both those present in the environment and in foods, by activating numerous genes. Many of these genes are directly or indirectly involved in determining the organism's virulence (Archer, 1996). This phenomena has been extensively studied in *Salmonella* spp. and *E. coli* . Mekalanos (1992) described stress-related virulence genes in different microorganisms, including *L. monocytogenes*.

Strains of *L. monocytogenes* vary in their degree of virulence (Meyers and Martin, 1994). Recent research suggests that the production of LLO is influenced by the growth environment. The virulence of *L. monocytogenes* increases when cells are grown at refrigeration temperatures (Meyers and Martin, 1994). Dallmier and Martin (1990) reported that growth in the presence of NaCl resulted in an increase in the activity levels of CA, SOD and LLO. Datta and Kothary (1993) demonstrated that temperature and pH altered LLO production. Production of LLO in *L. monocytogenes* decreased when the cells were grown at 26° rather than 36°C. LLO production was optimum at pH 7.0 and decreased with a decrease in pH.

Even though environmental stresses can influence the production of LLO, there is still debate over whether cells demonstrating an increased LLO production also demonstrate increased virulence in the host. In experimentally infected mice, increased

LLO production did not result in an increase in virulence. Meyers and Martin (1994) reported that growth at reduced temperatures did not influence virulence for mice infected i.g. or i.p. However, one study demonstrated an increased virulence in mice, when *L. monocytogenes* grown at 4°C was administered i.v. but not i.g. Changes in the environmental temperature probably do not significantly increase the virulence of foodborne infections of *L. monocytogenes*. It appears that the conditions of growth after infection may be more important contributors to virulence (Myers and Martin, 1994).

One environmental stress which could potentially affect the virulence of *L. monocytogenes* in the host is pH. When *L. monocytogenes* is first exposed to a mild acid stress, it demonstrates an increase lethality in mice relative to that of the wild type when inoculated by the interperitoneal route (O'Driscoll et al., 1996). Prolonged exposure of *L. monocytogenes* to strong acid conditions (pH 3.5) resulted in mutants which could constitutively demonstrate increased acid tolerance at all stages of the growth cycle. When acid adapted strains were administered i.g. to mice at a low inocula, increased numbers of these strains were recovered from the spleens of infected mice in greater numbers than found in mice infected with a high inocula of wild type strains. The increased capacity of acid tolerant *L. monocytogenes* to grow in pH range of 4.4 to 5.7 may aid in its passage through the stomach and its transient residence in the macrophage phagosome (O'Driscoll et al., 1996).

C. Biofilms

Bacterial cells associated with solid surfaces can result in the formation of biofilms. Biofilms are characterized as attached microorganisms (single or multiple species) organized within extensive exoploymer glycocalices. The exoploymer composition is not well defined but is thought to consist largely of polysaccharides. Other substances, organic and inorganic, may also be entrapped in the biofilm matrix. Biofilms are capable of forming in many different environments (marine environments, water pipelines, heat exchangers, cooling towers, implanted medical devices). In recent years, several reviews have been published emphasizing the importance of biofilm to the food industry (Hood and Zottola, 1995; Wong and Cerf, 1995).

Cells organized as biofilms are known to have an increased resistance to antimicrobial agents above that of their unattached (planktonic) counterparts. Recently, research has been directed towards explaining why microorganisms within biofilms are more resistant than planktonic cells. It has been suggested that the surrounding exopolymer (glycocalyx) of the biofilm excludes and or influences the access of antimicrobial agents to the cell surface of the underlying microorganisms. Though there are reductions in the diffusion coefficients of antimicrobial agents across the glycocalyx, these reductions alone are thought by some researchers to be insufficient to account for the observed recalcitrance (Brown and Gilbert, 1993).

As a barrier, the glycocalyx not only excludes antimicrobial agents but also limits the availability of key nutrients to organisms within a biofilm. Limitation of nutrients results in slow growth rates and the development of phenotypes which differ from planktonic cells. Changes in the growth rate are often accompanied by alterations in the cell envelope components. Specifically, these are changes in fatty acids, phospholipids, metal cations, envelope proteins and extracellular enzymes. These changes in turn influence the susceptibility of the cell towards antimicrobial agents. Biofilm recalcitrance might therefore relate to the heterogeneity of nutrient deprived phenotypes within the biofilms (Brown and Gilbert, 1993). The increased resistance of microbial biofilms to antimicrobial agents may, in part, be explained as a starvation stress response similar to the stress response demonstrated in planktonic organisms.

In addition to increased or altered protein synthesis, starvation stress is accompanied by an increase in cell number, a decrease in cell size, an increase in cell hydrophobicity and an increase in adhesiveness. One approach for in vitro biofilm development consists of an incubation in low-nutrient media for 36 h, followed by resuspension of harvested cells in a phosphate buffer for 4 h in the presence of the solid surface (Frank, 1993). It should be noted that when Jenkins et al., (1988) studied the cross protective response of planktonic *E. coli* following starvation, they found starvation for 4 h provided the maximum protection to later stresses. Other methods may vary slightly, however, most utilize a low nutrient media for cell growth followed by adherence to a solid surface in a phosphate buffer.

Ren and Frank (1993) have reported on the susceptibility of starved stressed planktonic and biofilm *L. monocytogenes* to a quaternary ammonium sanitizer (benzalkonium chloride). They concluded that starvation of *L. monocytogenes* is not consistently associated with an increased resistance to benzalknoium chloride. These researchers suggest that the ability of *L. monocytogenes* to survive in food plant environments is attributed to biofilm formation versus the effects of starvation stress. However, Leyer and Johnson (1993) suggested that changes in the outer membrane properties of *S. typhimurium* due to acid adaptation, including an increase in hydrophobicity, could affect the ability of the cell to attach to hydrophobic surfaces and form biofilms. Thus biofilm formation may be a result of an environmental stress induced response. It should be noted that the growth conditions used by Ren and Frank (1993) to induce biofilm formation are similar to those conditions used to induce starvation associated stress responses in the studies cited above. In an earlier study, Lee and Frank (1990) reported on the effect of growth temperature and media on the inactivation of *L. monocytogenes* by chlorine. They found that cells grown in low nutrient media at 35°C were more resistant to 5 ppm free chlorine than cells grown at the same temperature in trypticase soy broth. It is possible that the attached bacteria (starvation stressed) could have an increased resistance to other environmental strêsses including the byproducts of competing microflora.

D. Bacterial Attachment

1. Bacterial attachment models

One of the initial processes leading to biofilm formation is the adhesion of bacterial cells to solid surfaces. Biofilms are capable of forming on a variety of food contact surfaces which vary in their surface properties (i.e. hydrophobic, hydrophilic). There are 2 primary models proposed, a 2 step and a 3 step process, which describe how microorganisms attach to solid surfaces. The 2 step process (Marshall et al. 1971) involves the transport of bacteria close enough to the surface so that they can be adsorbed. This first step is reversible in that the bacteria can be removed by simple washing. In the second step, which is irreversible, the bacteria are anchored to the surface by extracellular material produced by the bacteria. The 3 step model of Busscher and Weerkamp (1987) describes attachment of bacteria in terms of the distance the microorganism is located from the surface. Distances greater than 50 nm involve long-range forces. At this distance, attachment is reversible. As the distance between the microorganism and surface approaches 20 nm, electrostatic interactions become involved. This step is at first reversible but, over time, is thought to become irreversible. The final step takes into account distances less than 15 nm, in which additional forces to those in the first 2 steps come into play. The production of adhesive polymers occurs which results in the irreversible attachment of the microorganism to the surface. In both models, the

occurrence of irreversible attachment is dependent upon the metabolic activity of the microorganism and its ability to produce adhesive materials.

2. Mechanisms of attachment

Current theories on the mechanisms by which microorganisms attach to solid substrata include: (1) receptor concept involving interactions between components of the cell and the substratum as in lectin-like interactions, (2) colloidal stability theories involving overall surfaces properties such as hydrophobicity and charge interactions, and (3) thermodynamic approaches based on interfacial free energies (Busscher and Weerkamp, 1987). Attachment of microorganisms to surfaces is metabolically favorable because organic molecules are found to be more concentrated at an interface. When surfaces are immersed in an aqueous environment, macromolecules and other low-molecular-weight, hydrophobic molecules adsorb to the surface to form a conditioning film. Conditioning films alter both the charge and free energy of the surface. It is not clear to what extent the bacterial cell wall interacts with the conditioning film. The number and type of bacteria that will adhere to a surface, as well as the strength of the attachment, varies with the type of the surface. Surfaces generally are placed into 1 of 2 categories (Lappin-Scott et al., 1992):

1. The high surface energy materials that are hydrophilic often carrying a negative charge (e.g., glass, metals, or mineral).
2. The low surface energy materials that are more hydrophobic with low charge (either positive or negative). Such surfaces include organic polymers such as plastics and Buna-N rubber.

Surfaces with a higher surface energy and activity more readily adsorb dissolved solutes or nutrients. The amount of nutrients at the surface then in turn affects the rate of bacterial colonization.

Pseudomonas spp. were found to attach in large numbers to hydrophobic plastic with no surface charge (Teflon and polystyrene), in moderate numbers to hydrophilic metals with a positive charge (platinum) and only in small numbers to hydrophilic, negatively charged substrata (glass) (Fletcher and Loeb, 1979). *Salmonella typhimurium* and *L. monocytogenes* both attach in large numbers to stainless steel and Buna-N rubber. However, when these surfaces were pretreated with milk proteins, attachment of these two organisms to either surface was significantly reduced (Helke et al., 1993). These researchers suggested that the milk proteins tested carried a net negative charge in their suspending menstruum, resulting in a repulsion of the negatively charged proteins adsorbed onto the surface and the negatively charged bacterial cells. Other researchers have reported on the effect of milk proteins on attachment of milk-associated microorganisms. Speers et al. (1984) observed an increase in attachment of milk-

associated microorganisms to stainless steel and rubber surfaces in the presence of whey proteins. Meadows (1971) reported an increase in attachment of gram negative bacteria to a hydrophilic surface in the presence of casein.

Bacteria have been referred to as living colloids because of their small size, low density, net negative charge and variable degrees of cell surface hydrophobicity. Colloidal chemistry (DLVO theory) has been used to explain some of their behavior at or near surfaces (Pethica, 1980) . Based on colloidal chemistry, bacteria should be repelled electrostatically from surfaces at very low electrolyte concentrations. At higher concentrations, Van der Waals attractive forces can exceed electrical repulsion forces at distances of 10 to 20 nm from the surface. However, the strong repulsion forces should still prevent a body as large as a bacterium from making direct contact with a surface (Busscher and Weerkamp, 1987). The behavior of bacteria at or near a surface can not be fully explained by chemistry but must also consider any biological responses of the cell that may be involved in attachment. It has been proposed, that the strong repulsive forces, which prevents the bacterium from making direct contact with the surface, are overcome by the production of extracellular polymeric substances (EPS) (Marshall, 1992). Because these substances are not subject to these same repulsive forces, they are able to form a bridge between the bacterium and a food contact surface. This polymer bridging involves combinations of chemical bonding (electrostatic, covalent, and hydrogen), dipole interactions and hydrophobic interactions (Marshall, 1992).

The composition and quantity of bacterial surface polymers vary considerably and are strongly influenced by growth and environmental conditions. Although extracellular polymers have often been reported as responsible for irreversible adhesion, this is not always the case. Brown et al. (1990) demonstrated widespread adhesion from mixed populations in a carbon-limited culture, despite no obvious evidence of extracellular polymeric production. A nitrogen-limited culture, however, resulted in poor adhesion, although large extracellular polymeric substances were observed.

Paul and Jeffery (1985) studied adhesion of *Vibrio proteolytica* to hydrophobic and hydrophilic surfaces. These researchers found that the attachment of *V. proteolytica* to a hydrophobic substratum (polystyrene) could be inhibited by greater than 97% by the proteolytic enzymes trypsin, pronase and chymotrypsin. These same enzymes were ineffective in preventing attachment of *V. proteolytica* to hydrophilic substrata such as glass or tissue culture dishes. These researchers suggested the existence of separate mechanisms for the adhesion of *V. proteolytica* to hydrophobic and hydrophilic substrata.

The nature of the surface, in part, dictates the degree to which microorganisms will attach to that surface, since the same microorganism will attach to different surfaces with varying degrees of efficiency. Numerous researchers have investigated the effect of bacterial surface hydrophobicity on the ability of bacteria to attach (Allison et al., 1990; Mafu et al., 1991). Methods used to determine hydrophobicity include, salt aggregation, electrophoretic mobility, bacterial adherence to hydrocarbons, hydrophobic interaction chromatography (HIC) and contact angle. Using the contact angle method, Mafu et al.

(1991) determined that the surface free energy of *L. monocytogenes* Scott A is 65.9 mJ · m^{-2} , classifying the microorganism as a hydrophilic organism. The hydrophilic nature of the bacterial surface was furthered confirmed using the salt aggregation and HIC. *L. monocytogenes* aggregated at very high ammonium salt molarities and did not bind to octyl-sepharose using the HIC method.

Based on the thermodynamic concept proposed by Absolom et al. (1983) and Busscher et al. (1987), adhesion of *L. monocytogenes* should be more energetically favorable as the surface free energy of the solid substratum decreases. However, Mafu et al. (1991), did not find correlation between the low surface energy of polypropylene and rubber and the ability of *L. monocytogenes* to attach. In fact, these researchers found that *L. monocytogenes* could attach to both low and high energy surfaces after short contact times, suggesting that factors other than cell surface hydrophobicity is involved in the adhesion process of this microorganism.

3. Physiological response of attachment

Surface-induced stimulation of bacterial activity is well documented. Changes may include smaller cell size or an increased or altered production of extracellular material. The environment at the surface may differ from that of the bulk phase (e.g., pH, redox potential) eliciting a response from the microorganism. Vandevivere and Kirkman (1993) reported that attachment to a solid surface induces exopolymer production by a number of subsurface isolates. When these same isolates were resuspended in fresh medium,

exoploymer production decreased. The increase in exoploymer synthesis did not involve changes of the specific growth rate, growth stage or limiting nutrient. Much debate exists, however, about whether such stimulation results from derepression/induction, by contact with the surface, through specific operons/genes or is indirect and reflects physio-chemical influences of the surface on the surroundings of the cell wall (van Loosdrecht et al., 1990).

Under different conditions, the change observed in attached cells may be part of a sequence which begins before adhesion with cells in the planktonic state. In such cases, the changes in the cell may influence adhesive properties. The physiological state of the organism influences the rate and, possibly, the extent of attachment. Bryers and Characklis (1982) observed that attachment rate was directly proportional to growth rate in a mixed culture system. Others have reported a decrease in attachment rate with increasing specific growth rate for *Pseudomonas*. Pickett and Murano (1996) investigated the affect of chemical shock on the ability of *L. monocytogenes* to attach to stainless steel. They reported attachment levels of *L. monocytogenes* to be 10^3 CFU/ml for both chemically treated and non-treated cells after a 24 h exposure period. Because attachment levels were not determined for exposure times shorter than 24 h, it is not known whether chemical shock affected the rate of attachment. Proteins induced by environmental stresses may participate in attachment. Harold and Zottola (1989) have suggested that bacterial attachment to stainless steel surfaces involves a polysaccharide and protein matrix. These researchers observed that trypsin was effective in removing attached cells of *P. fragi*.

4. Adsorption kinetics of microbial adhesion

Attachment is dependent upon the chemical and physical interactions between the potential substratum and the bacterial surface (Fletcher and Loeb, 1979). Physical chemistry has long been used to study the adsorption of gases onto solid surfaces (e.g., Langmuir equation). However, these theories have typically been applied only to the adhesion of "ideal particles". Microorganisms differ from these "ideal particles" in that, they are constantly changing to respond to their environment. Cells have neither a simple geometry nor a simple, uniform molecular composition. Intracellular chemical reactions are constantly occurring that affect the molecular composition both in the interior and at the surface of the microorganism. Application of thermodynamic principles to microbial adhesion is further complicated by the legitimacy of whether equilibrium conditions are achieved (Rutter and Vincent, 1984). Equilibrium conditions are only achieved in a closed system where no exchange of matter or energy occurs with the surroundings. Biological systems are primarily open systems, in which a steady state at best may be realized.

Even with these recognized limitations, the application of physical chemistry has proven useful in the study of bacterial attachment. Dabros and van de Ven (1983) found a number of similarities between the adhesion of bacterial cells and polystyrene latex particles. The latex particles, which are negatively charged, like a large majority of bacterial cell surfaces, adhered to negatively charged glass. Vanheacke et al. (1990) applied kinetic equations, developed for the adsorption of small particles onto inert surfaces, to describe adhesion of *Pseudomonas aeruginosa* to stainless steel. For both the

adhesion of microorganisms and the adhesion of inert particles, the importance of desorption as well as adsorption was emphasized. Many studies on microbial attachment and biofilm formation have arbitrarily defined irreversibly attached cells as those cells which remained attached after simple washing. Irreversible attachment can be better described using thermodynamic principles. A particle is irreversibly attached to a surface when the free energy minimum (G_{min}) involved in the particle-surface interaction is large [i.e., G_{min}>10kT, for example], where k is the rate of the reaction and T is the temperature at which the reaction occurs (Rutter, and Vincent, 1984).

The Langmuir (1918) equation describes the adsorption of gases onto solid surfaces, for example, a surface having a certain number of adsorption sites S of which S_1 may be occupied by the adsorbate. With S_0, representing unoccupied sites, $S_0 = S - S_1$. Adsorption equilibrium is treated as a dynamic state in which the rate of adsorption is equal to the rate of desorption. The rate of adsorption (k_2) is proportional to the pressure (P) and surface collision frequency of the gaseous adsorbate on the unoccupied sites:

$$\text{rate of adsorption} = k_2PS_0 = k_2P(S - S_1)$$

The rate of desorption (k_1) is proportional to the number of occupied sites S_1:

$$\text{rate of desorption} = k_1S_1$$

Setting the rate of adsorption equal to the rate of desorption, and solving for S_1 gives

$$\frac{S_1}{S} = \theta = \frac{bP}{1 + bP}$$

where θ is the fraction of surface covered and $b = k_2/k_1$.

Relatively few chemisorption systems obey the simple Langmuir model well, especially at the extremes. The basic model, however, is sound. Often, deviations from the model are attributed to nonuniformity of the test surface. For irreversibly bound cells, the kinetic analysis proposed by Langmuir in the context of gas adsorption may be adopted, where the number of small particles as a function of time n(t) is written as:

$$\frac{dn(t)}{dt} = -k\, n(t)\, [1-\theta\,(t)]$$

where k is the rate constant, and θ (t) is the coverage of the macroscopic surfaces, as given by:

$$\theta\,(t) = \frac{n_0 - n(t)}{n_0 - n_f}$$

where n_0 and n_f are the initial and final number concentrations of the particles in suspension, respectively. This adsorption model is only applicable when attachment is irreversible. For discussions on reversible adhesion see Vincent et al. (1980) and Boughley et al. (1978).

5. Detachment from the surface

Traditionally, detachment of microorganisms from surfaces has been studied in mature biofilms. The detachment phenomena, as it applies to microorganisms within a biofilm, may be categorized as shearing or sloughing. Shearing refers to the continuous removal of small portions of the biofilm. This category of detachment is dependent on fluid dynamic conditions. With an increase in biofilm thickness and fluid shear stress at the biofilm-fluid interface, the rate of detachment also increases. Sloughing refers to a random, massive removal of biofilm attributable to changes within the biofilm environment such as fluctuations in nutrient or oxygen levels. Detachment has also been shown to occur with various chemical treatments. Treatments that have been effective in removing biofilm material from surfaces include: (1) oxidizing biocides (e.g., chlorine), (2) UV radiation, (3) surfactants, and (4) non-oxidizing biocides (Characklis, 1984). Herald and Zottola (1989) found that trypsin, sodium dodecyl sulfate (SDS) and NaOH were effective in removing cells of *Pseudomonas fragi* attached overnight to stainless steel. However, when the surface was treated with Cetavolon, a chemical known to react with polysaccharides, detachment did not occur. Addition of Triton-X, a nonionic surfactant, to the surface resulted in stabilization of attached *P. fragi*.

Over the years, the most frequently used methods to enumerate bacteria on surfaces include agar flood method, rinse solution method and agar contact method. It has been noted, that when any one of these methods is repeatedly applied to the same area

on a surface the number of bacteria removed decreases with each application. Mathematically this has been expressed as

$$N_T = N_1/(1 - 10^s)$$

where N_1 is the number of colonies obtained at the first sampling and s is the slope of the line obtained when log CFU is plotted against the sample number. Given a linear relationship is obtained, the total number of bacteria that can be removed from the surface, N_T, can be calculated (Wong and Cerf, 1995).

To date, few reports have been published on the removal of attached microorganisms that have yet to form a mature biofilm. However, detachment of microbial cells and related biofilm material occurs from the moment of initial attachment (Characklis, 1984). The ease with which microorganisms shed from surfaces contributes to their ability to spread and contaminate food product items in their vicinity (Eginton et al., 1995). Food-borne pathogens possessing a low infective dose, such as *E. coli* O157:H7, or pathogens currently regulated as zero tolerance in ready-to-eat foods, such as *Salmonella* spp. and *Listeria monocytogenes*, do not have to be present on food contact surfaces at levels consistent with mature biofilms to present a public health threat. A better understanding of the factors affecting the strength of attachment of microorganisms to food contact surfaces is needed.

6. *Listeria* and attachment

Herald and Zottola (1988) first studied the attachment of *L. monocytogenes* to a food contact surface. Using scanning electron microscopy (SEM), these investigators demonstrated the ability of *L. monocytogenes* to attach to stainless steel at various temperatures and pH values. Other researchers have used similar SEM techniques to describe *L. monocytogenes* attachment to various food contact surfaces such as stainless steel, polypropylene, and rubber (Mafu et al., 1990). Recently, Blackman and Frank (1996) demonstrated that *L. monocytogenes* could attach and form single species biofilms on various food processing surfaces including stainless steel, Teflon®, nylon, and polyester floor sealant. However, research efforts have largely concentrated on determining the resistance of *L. monocytogenes* (either attached or within a biofilm) to cleaners and common sanitizers (Frank, 1990; Krysinski et al., 1992; Lee and Frank, 1991; Ren and Frank, 1993). To date, few if any reported studies have addressed the effect of environmental stresses on the ability of *L. monocytogenes* to attach to different surfaces. The capability of *L. monocytogenes* to adhere to surfaces represents a source of potential contamination for a wide variety of material coming in contact with solid surfaces. However, the environmental conditions which affect the rate of adhesion of *L. monocytogenes* to food contact surfaces are not well understood and have not been defined.

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III. EFFECT OF ENVIRONMENTAL STRESS ON THE ABILITY OF *LISTERIA MONOCYTOGENES* SCOTT A TO ATTACH TO FOOD CONTACT SURFACES

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ABSTRACT

Attachment of *Listeria monocytogenes* Scott A to Buna-N rubber and stainless steel under different temperature and pH conditions at the time of cell growth or at the time of attachment were investigated. All experiments were conducted in sterile phosphate buffer to avoid cell growth during exposure to the test surfaces. Numbers of attached cells increased with increasing attachment temperature (10° to 45°C) and exposure time for both test surfaces. Maximum levels of attached cells were obtained when cell growth occurred at 30°C. Downward, but not upward, shifts in the cell suspension holding temperature prior to attachment to Buna-N rubber resulted in reduced adhered cell populations. Maximum levels of adhered cells to Buna-N rubber were not affected by adjustments of the attachment medium pH between 4 to 9. However, after short contact times (i.e. less than 30 min), levels of attached cells were lower when attachment occurred under alkaline conditions. Growth pH was also found to affect the levels of adhered cell populations to Buna-N rubber. *L. monocytogenes* Scott A was found to attach at higher levels on stainless steel than Buna-N rubber for all temperature and pH parameters evaluated in this study. Greater differences in attached cell populations were also observed for Buna-N rubber during the first 30 min of exposure when compared to stainless steel for all temperature and pH conditions investigated.

INTRODUCTION

Both physical and chemical treatments are used in food processing to eliminate and control the presence of pathogenic and spoilage microorganisms in foods. When cleaning and sanitizing regimes are poorly executed or inadequate, microorganisms may remain in the processing environment in an injured state. sublethal treatments or stresses (i.e. heat, anaerobiosis, oxidation, starvation, cold shock, ethanol) can result in unique adaptive responses by bacteria, including those of public health concern (*3,7,13,17,18,21,25,31*). For example, starvation stress is often accompanied by an increase in cell number, a decrease in cell size, an increase in cell surface hydrophobicity and an increase in adhesiveness. these factors make the bacteria more prone to adhere to solid surfaces where they benefit from an enhanced nutrient status (*20*). Nystrom et al. (*24*) demonstrated increased adherence of *Vibrio* spp. to a variety of surfaces after nutrient depletion. Continued adhesion and growth of adhering bacteria eventually leads to biofilm formation.

L. monocytogenes is ubiquitous in nature, being found on soil, feces, plants and in water samples (*22*). Consequently, this pathogen is frequently found on raw materials used in food processing. Cox et al. (*5*) demonstrated that listeriae could be found in all types of food production environments. Once present in the processing environment, control of *L. monocytogenes* has proven to be difficult. Although improvements have

been made in controlling *L. monocytogenes* in food systems, its presence in the processing environment is still readily found.

A number of studies have demonstrated the ability of *L. monocytogenes* to attach and form biofilms on food contact surfaces (*2,11*). To date, the majority of research in the food industry addressing adhered *L. monocytogenes* has focused on the ability of this microorganism to resist environmental stresses (*8,12,15,16,26*). Herald and Zottola (*11*) first studied the attachment of *L. monocytogenes* to a food contact surface. Other researchers have used similar SEM techniques to describe *L. monocytogenes* attachment to various food contact surfaces such as stainless steel, polypropylene, and rubber (*2,19*). However, only a few studies have addressed what affect environmental stresses have on the ability of *L. monocytogenes* to attach to different surfaces. The capability of *L. monocytogenes* to adhere to surfaces represents a source of potential contamination for a wide variety of material coming in contact with solid surfaces. The environmental conditions which affect adhesion of *L. monocytogenes* to food contact surfaces are not well understood. Therefore, the objective of this study is to determine, in a quantitative manner, the effects of different environmental conditions on the ability of *Listeria monocytogenes* Scott A to food contact surfaces.

MATERIALS AND METHODS

Test organism and culture maintenance

L. monocytogenes strain Scott A was obtained from the culture collection of the Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg VA. The culture was maintained on trypticase soy agar (TSA, Difco, Detroit, MI) supplemented with 0.6% yeast extract (YE, Difco) slants (TSA-YE) held at 4 °C. Prior to use, the culture was subcultured on 2 consecutive days in trypticase soy broth (TSB, Difco) supplemented with 0.6% yeast extract (Difco) (TSB-YE) and incubated at 37 °C.

Test surfaces

Buna-N rubber, obtained from M. G. Newell Greensboro, NC, and stainless steel (type 304, #4 finish), were used for attachment of *L. monocytogenes*. The test surfaces were cut into 2 x 5 cm coupons (ca. 2 mm in thickness), washed by soaking in warm alkaline cleaner (Klenzade, Division of Ecolab Inc., St. Paul, MN) overnight and rinsed repeatedly with distilled water. The surfaces were sterilized by autoclaving for 15 min at 121°C .

Attached cell development

One ml of a 18-24 h TSB-YE culture was used to inoculate 250 ml of TSB-YE pre-tempered to the growth temperature. Cells were incubated at the growth temperature with shaking (100 rpm) and grown to mid-log phase based on previous growth curve studies. Mid-log phase cells were harvested by centrifugation, washed and resuspended in an equal volume of sterile phosphate buffer (0.02 M Potassium Phosphate Buffer, pH 7.0) (PB). One ml aliquots of the washed cell suspension were used to inoculate multiple 250 ml screw cap flasks containing 99 ml of PB pre-tempered to the attachment temperature to give a target level of 1 x 10^7 CFU/ml. After incubation for 15 min with shaking (100 rpm) at the attachment temperature, 1 sterile Buna-N rubber or stainless steel coupon pre-tempered to the attachment temperature was added to each flask. Incubation with shaking was continued at the test temperature. Coupons for each test surfaces were removed after 0, 5, 10, 15, 20, 30, 60, and 120 min of exposure. Test surfaces were prepared for enumeration and attached cells were quantitated as described below.

Enumeration of attached cells

To enumerate attached cell populations the test surfaces were first rinsed in 20 ml volumes of the sterile PB. This rinse step was repeated 4 more times to remove any reversibly attached cells. Viable counts on the rinse buffer established that less than 1 CFU per ml was detected in the fifth rinse. After the last rinse, the buffer was aspirated and the

side of the surface which had been positioned upward in the flask during exposure to the cell suspension was swabbed with a calcium alginate swab. The swab was transferred to 10 ml of sterile PB and the sample serially diluted in 0.1% peptone. Attached cells were enumerated on TSA-YE spread plates incubated at 37°C for 48 h. Duplicate surfaces were enumerated at each attachment period and the average number of cells was calculated as CFU per cm^2 test surface. Swabbed surfaces were pour plated with TSA-YE and incubated at 37°C for 48 h to ensure that all attached cells had been removed.

Effect of temperature on attachment

Three sets of experiments were performed to determine the effect of temperature on the ability of *L. monocytogenes* to attach to the test surfaces. The first set determined the effect of growth temperature prior to attachment. Cells were grown to mid-log phase in TSB-YE at either 10°, 30° or 42°C with shaking. For each growth temperature, test surfaces (Buna-N rubber only) were added to test cell suspensions at 30°C as previously described. Triplicate trials were performed for each growth temperature.

The second set of experiments was designed to determine the effect of temperature at the time of attachment. In these experiments, cells were grown to mid-log phase at 30°C. Test cell suspensions were prepared as previously described and held for 15 min at either 10°, 30° or 45°C prior to the addition of Buna-N rubber surfaces. Stainless steel surfaces were tested at attachment temperatures of 10°, 30°, and 45°C only. After exposure for the pre-determined times, surfaces were removed and attached cells enumerated. Triplicate trials were performed for each attachment temperature.

The third set of experiments was performed to determine the effect of shifts in temperature on the ability of *L. monocytogenes* to attach to Buna-N rubber surfaces. In these experiments, cells were grown to mid-log phase in TSB-YE at 30°C. Ten ml of the washed cell suspension was used to inoculate flasks containing 90 ml of PB pre-tempered to 37°C. After 15 min at 37°C, 10 ml aliquots were removed and used to inoculate flasks containing 90 ml of PB pre-tempered to 45°C. The Buna-N rubber surfaces were immediately added and held for the pre-determined exposure times prior to enumeration. This experiment was then repeated where cells were grown to mid-log at 30°C, shifted to 21°C and attached at 10°C. Triplicate trials were performed for each temperature shift. The cell concentration in the test suspensions for all experimental trials was maintained at approximately 1 x 10^7 CFU/ml.

Effect of pH on attachment

The following experiments were performed to determine the effect of pH on the ability of *L. monocytogenes* to attach to the test surfaces. The first set of trials were designed to determine the effect of growth pH prior to attachment. Cells were grown to mid-log phase at 30°C in TSB-YE adjusted to either pH 5.5, 7.0 or 8.5. For each growth pH, the test surfaces were added to the cell suspension in PB (pH 7.0) and incubated at 30°C as previously described. Triplicate trials were performed for each growth pH.

Cells were grown to mid-log phase at 30°C in TSB-YE (pH 7.0) to determine the effect of pH at the time of attachment to Buna-N rubber. Test cell suspensions were

prepared in sterile PB adjusted to either pH 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0. After addition and exposure for the pre-determined times, surfaces were removed and attached cells enumerated. Triplicate trials were performed at each attachment pH level.

RESULTS

Effect of temperature on attachment

Cells of *L. monocytogenes* Scott A were able to attach to Buna-N rubber and stainless steel at all temperatures evaluated (Figures 1 and 2). The numbers of attached cells increased with increasing temperature and exposure time for both Buna-N rubber and stainless steel. During the first 10 min of exposure, levels of attached *L. monocytogenes* were consistently higher on stainless steel across all test temperatures. Attached cell populations at 45°C and 10°C differed by approximately 1 log on Buna-N rubber and 0.5 log on stainless steel after exposure for 120 min. A comparison of the maximum number of adhered cells obtained for the 2 test surfaces at the different attachment temperatures indicated that higher levels of attachment occurred on stainless steel (Figure 3). When the temperature for cell attachment was decreased, the difference in the maximum number of adhered cells detected on the 2 different surfaces was shown to increase.

The effect of growth temperature on attachment of *L. monocytogenes* to Buna-N rubber at 30°C was determined (Figure 4). Cells grown at 30°C prior to exposure to the test surface resulted in the highest concentration of attached cells, irrespective of the exposure time. The lowest concentration of attached cells, after 120 min of contact with the test surface, was observed for cells grown at 10°C. Cells grown at 42°C were found to have adhered cell populations similar to 30°C grown cells at the end of the test period. However, the adhered population for 42°C grown cells was found to be approximately 1

log lower than that observed for 30°C grown cells during the first 10 min of exposure to Buna-N rubber (Figure 4).

The effect of shifts in temperature on the attachment of *L. monocytogenes* to Buna-N rubber was studied (Figure 5). An increase in temperature from 30° to 37°C for 15 min prior to exposure to Buna-N rubber at 45°C did not appear to affect the ability of *L. monocytogenes* to adhere to this surface. At all sampling times, similar attached cell populations at 45°C were obtained for both shifted and non-shifted cells. A decrease in temperature from 30° to 21°C for 15 min prior to attachment at 10°C did, however, result in attachment levels which differed from cells attached at 10°C with no prior temperature shift. Over the 120 min exposure period, the concentration of attached cells at 10°C was consistently lower for cells which were shifted to 21°C prior to attachment. Maximum attachment levels of non-shifted cells were approximately 2 logs higher than the maximum level obtained for shifted cells.

Effect of pH on attachment

L. monocytogenes was able to attach to Buna-N rubber at all pH values evaluated (Figure 6). The maximum levels of attached *L. monocytogenes* (grown in TSB-YE, pH 7.0 at 30°C) obtained on Buna-N rubber after the 120 min exposure period was not affected by altering the pH of the attachment environment within the pH range of 4 to 9. However, alkaline pH levels resulted in lower numbers of attached cells at short contact times when compared to neutral or acidic attachment conditions.

Attachment of *L. monocytogenes* to Buna-N rubber and stainless steel in PB (pH 7.0) after growth to mid-log in TSB-YE adjusted to either pH 5.5, 7.0 or 8.5 was determined (Figures 7 and 8). On Buna-N rubber, the highest concentration of adhered cells occurred for the pH 7.0 grown cells. The maximum level of attached cells, occurring after 120 min exposure time, was approximately 1 x 10^4 CFU/cm^2 for pH 7.0, 1 x 10^3 CFU/cm^2 for pH 8.5, and 1.5 x 10^2 CFU/cm^2 for pH 5.5 grown cells. After 5 min exposure time, the concentration of attached cells on Buna-N rubber ranged from 3.0 x 10^2 CFU/cm^2 to < 10 CFU/cm^2 for pH 7.0 and pH 5.5 grown cells , respectively. Similarly on stainless steel, the highest concentration of attached cells across all sampling times was obtained for pH 7.0 grown cells and the lowest concentration was obtained for pH 5.5 grown cells. Higher numbers of adhered cells were obtained on stainless steel for all growth pH values tested, regardless of the exposure time, when compared to Buna-N rubber (Figure 9).

DISCUSSION

Attachment is dependent upon the chemical and physical interactions between the potential substratum, the bacterial surface, and the polymeric adhesive (*6*). The influence of a number of surface properties, both substratum and bacterium, on attachment have been investigated (e.g., hydrophobicity, surface charge, surface free energy, and extracellular polymer production). Buna-N rubber and stainless steel were used in this study because of their widespread use in the food industry and because they differ in their physical properties. Buna-N rubber is hydrophobic and stainless steel is hydrophilic, though it is thought to possess hydrophobic regions. The original surface characteristics of a substratum can be altered by the aqueous environment. Surfaces are conditioned by adsorption of molecules from the surrounding bulk fluid. Adsorbed molecules, both organic and inorganic, in turn alter the surface properties of the substratum. Bacterial attachment to conditioned surfaces has been shown to differ depending upon the molecules adsorbed and the microorganisms present. Helke et al. (*10*) reported a decrease in the level of attachment of *L. monocytogenes* and *Salmonella typhimurium* in the presence of milk proteins. While others have reported an increase in attachment in the presence of whey proteins (*28*). Adhesion to food contact surfaces has also been reported in Ringer's solution (*30*) and pork liquor (*4*). It appears that conditioning of a surface with organic molecules is not required for adhesion to occur and may even result in reduced levels of microorganisms that initially attach to that surface (*12*).

In this study *L. monocytogenes*, suspended in PB, was able to attach to both test surfaces within short contact times. In addition, the time to attach and the extent of attachment to the surfaces was influenced by the environmental conditions. A number of researchers have previously demonstrated the ability of *L. monocytogenes* to attach to Buna-N rubber and stainless steel. Mafu et al. (*19*) observed attachment of *L. monocytogenes*, using SEM, to rubber and stainless steel after 20 min contact time. *L. monocytogenes* was shown to adhere to stainless steel, polypropylene, glass and rubber to varying degrees at both 20° and 4°C. Herald and Zottola (*11*) used SEM to demonstrate adherence of *L. monocytogenes* to stainless steel at different temperatures and pH levels. However, few if any attempts have been made to quantitate the levels of attachment to food contact surfaces under different environmental conditions.

Environmental conditions are known to affect cell surface characteristics. Growth rate as well as growth phase have been shown to influence the regulatory mechanisms which determine cell surface properties (*9*). Exposure to acid stress conditions has resulted in specific outer membrane proteins being produced in *S. typhimurium* which in turn caused an increase in cell surface hydrophobicity (*17*). Temperature has been widely reported to affect protein synthesis. Jones et al. (*14*) reported the synthesis of unique outer membrane proteins by *E. coli* when growth occurred at 10°C. These researchers suggested that the presence of new outer membrane proteins accounted for the increased stability of attached cells in the absence of an extracellular matrix.

In recent years, the majority of biofilm research within the food industry has focused on the resistance of microorganisms within biofilms to common sanitizers and other antimicrobials. Very little work has been directed toward identifying environmental variables which influence the attachment of microorganisms to food contact surfaces. A need for further investigations on the relative importance of extracellular polymers, metabolic rate, motility, pH , temperature, etc. on attachment of microorganisms to surfaces was suggested by Rutter and Vincent (*27*).

Evaluating both temperature and pH on the ability of *L. monocytogenes* to attach, we found that environmental conditions influenced the attachment of this microorganism to food contact surfaces. Temperature at the time of attachment affected the ability of *L. monocytogenes* to attach to both Buna-N rubber and stainless steel. For Buna-N rubber, cells grown at 30°C adhered to a greater extent when attachment occurred at 45°C. Lower levels of attachment occurred at 10°C when compared to the higher temperatures investigated. Others have reported increased attachment when conditions for metabolic activity are most favorable (*11,29*). The decrease in attachment at the lower temperature may, in part, result from a decrease in hydrophobic interactions between the cell surface and the hydrophobic surface of the Buna-N rubber. Differences observed in the attachment curves (Figures 1 and 2) at the high and low attachment temperatures were greater on Buna-N rubber than on stainless steel which would support the involvement of hydrophobic interactions.

Attached cell populations were highest for cells grown at 30°C when metabolic activity was optimum as compared to the other growth temperatures (Figure 4). The differences in adhered cell populations observed for cultures grown at 10°, 30° and 42°C cannot be attributed to growth phase, since all cells were grown to mid-log phase prior to attachment. The numbers of adhered cells may, however, reflect the growth rate of the microorganism. Growth rates have previously been shown to influence cell surface hydrophobicity. Allison et al. (*1*) demonstrated that the surface hydrophobicities of various Gram-negative species were directly related to growth rate.

No difference in attached cell populations was observed when test cell suspensions experienced an upward temperature shift (Figure 5). However when the temperature was shifted down, the extent of attachment occurring on Buna-N rubber was reduced when compared to cells which were attached at 10°C with no prior temperature shift. When growing cells are shifted to temperatures within the normal growth range, growth continues at a rate characteristic of the new temperature. However, when cells are shifted to an extreme high or low temperature, the growth rate decreases dramatically, sometimes even stopping altogether for a period of time (*23*). The differences in attachment observed in this temperature shift experiment may be a result of changes in growth rate. The shift from 30° to 10°C did not affect the attachment process (since attachment has been found to occur very rapidly) as much as for those cells which had undergone a shift to 21°C prior to exposure to the test surface. This temperature change may have resulted in an altered growth or metabolic rate which in turn affected the attachment process.

Herald and Zottola (*11*) reported higher numbers of attached cells to stainless steel at pH 7.0 and 8.0 when compared to pH 5.0. These researchers were using SEM to observe attached cells after contact to the test surface in TSB for 18-36 h. The increase in attachment was attributed to the optimum metabolic activity for this organism between pH 7.0 and 8.0. No attempts were made to determine the influence of growth rate or growth phase on attachment during these extended contact times. We found maximum levels of adhered cells on Buna-N rubber were reached within the 120 min exposure time and did not differ for all attachment pH conditions studied (Figure 6). However, differences in attached cell populations throughout the 120 min exposure were observed when cells were grown to mid-log at either pH 5.5, 7.0, or 8.5. Higher numbers of attached cells were detected on both surfaces when grown at a pH level optimum for metabolic activity (i.e. pH 7.0) Although effects of growth pH on adhered cell populations were observed on both test surfaces, a marked decrease in attachment to Buna-N rubber occurred when cells were first grown to mid-log at pH 5.5.

In this study, *L. monocytogenes* was found to attach at higher levels on stainless steel, irrespective of the environmental conditions. These findings are consistent with others who have used SEM techniques to demonstrate greater attachment to stainless steel when compared to Buna-N rubber (*11,19*).

In conclusion, we showed that exposing cells of *L. monocytogenes* to sublethal levels of environmental stress such as pH and temperature can affect the ability of this pathogen to attach to common food contact surfaces. These results indicate that

fundamental food processing parameters can influence the persistence of *L. monocytogenes* in the processing environment. A better understanding of those processing conditions which affect attachment of microorganisms is needed to prevent biofilm development. Further investigations into the affect of extreme environmental stress on the ability of pathogenic microorganisms to adhere to surfaces is warranted. Such studies could provide valuable insights into the attachment mechanisms and perhaps lead to better methods of control.

ACKNOWLEDGMENTS

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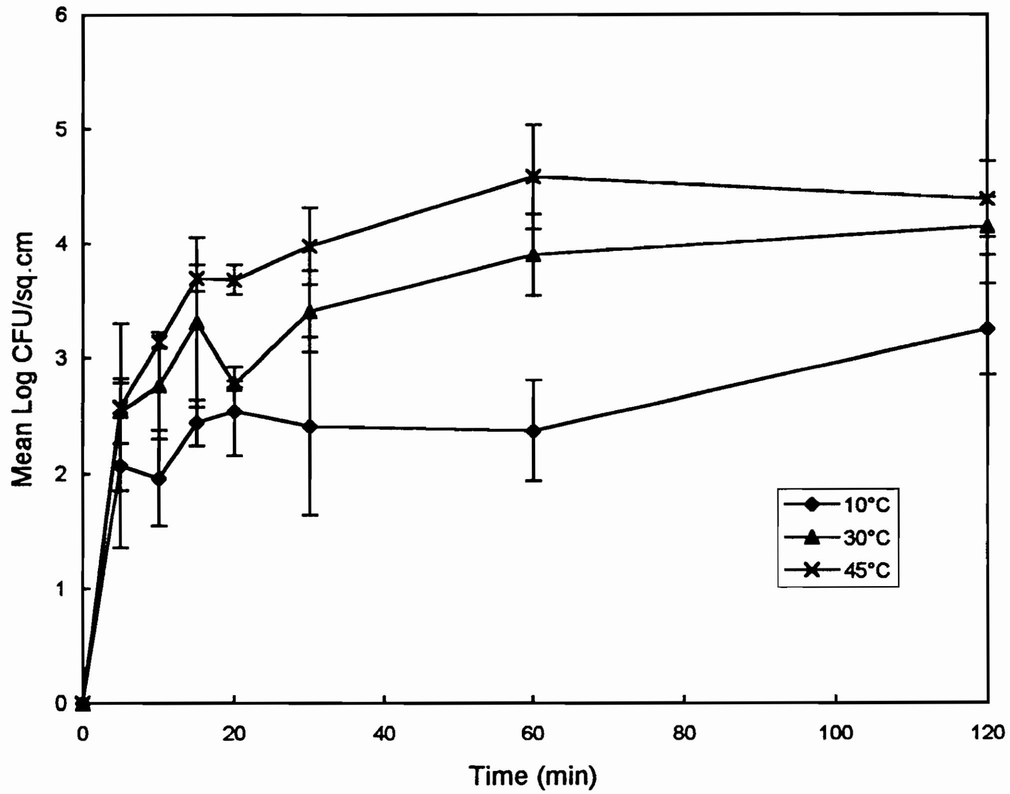

Figure 1. Attachment profile for Listeria monocytogenes *Scott A grown to mid-log at 30°C in TSB-YE (pH 7.0) and exposed to Buna-N rubber at 10°, 30°, and 45°C in PB (pH 7.0) for 120 min.*

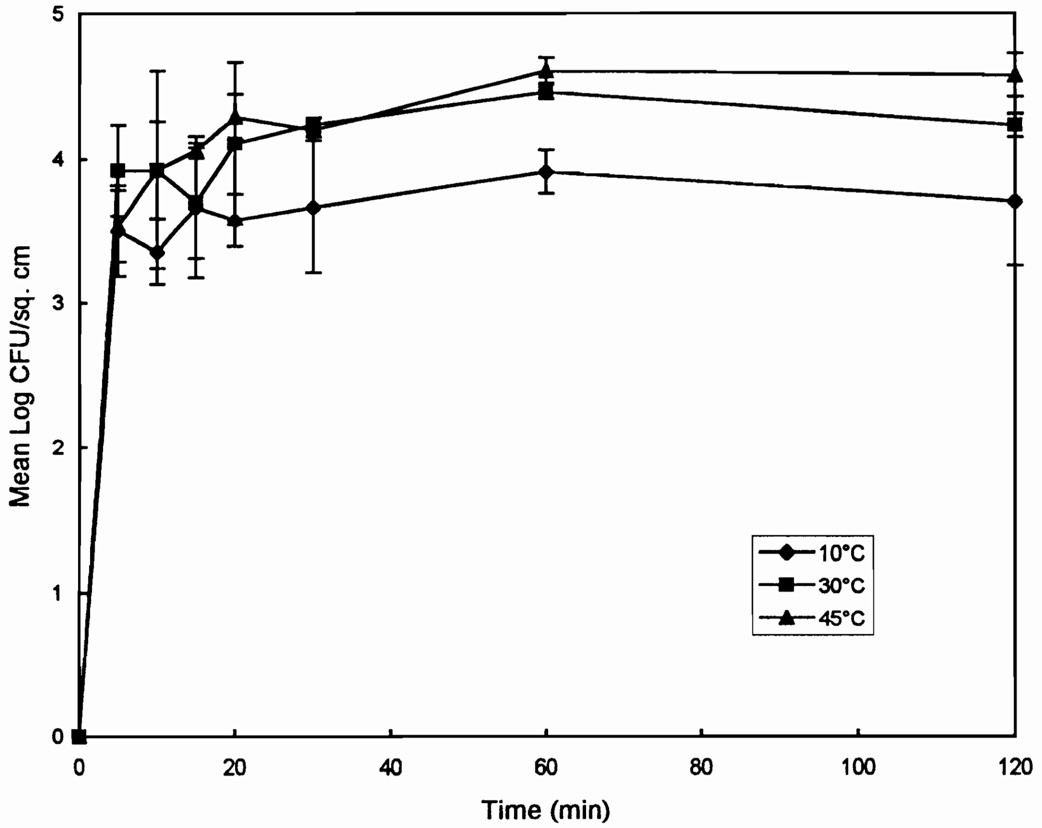

Figure 2. Attachment profile for Listeria monocytogenes *Scott A grown to mid-log at 30°C in TSB-YE (pH 7.0) and exposed to stainless steel at 10°, 30°, and 45°C in PB (pH 7.0) for 120 min.*

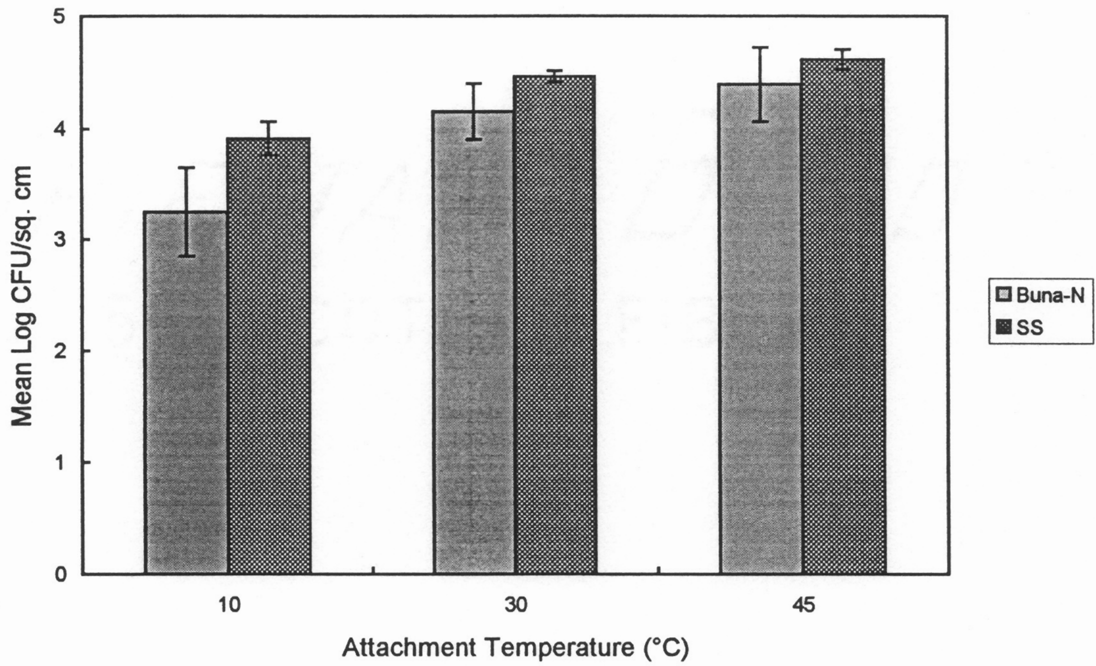

Figure 3. Effect of attachment temperature on maximum number of Listeria monocytogenes *Scott A detected on Buna-N rubber and stainless steel surfaces after 120 min exposure in PB (pH 7.0).*

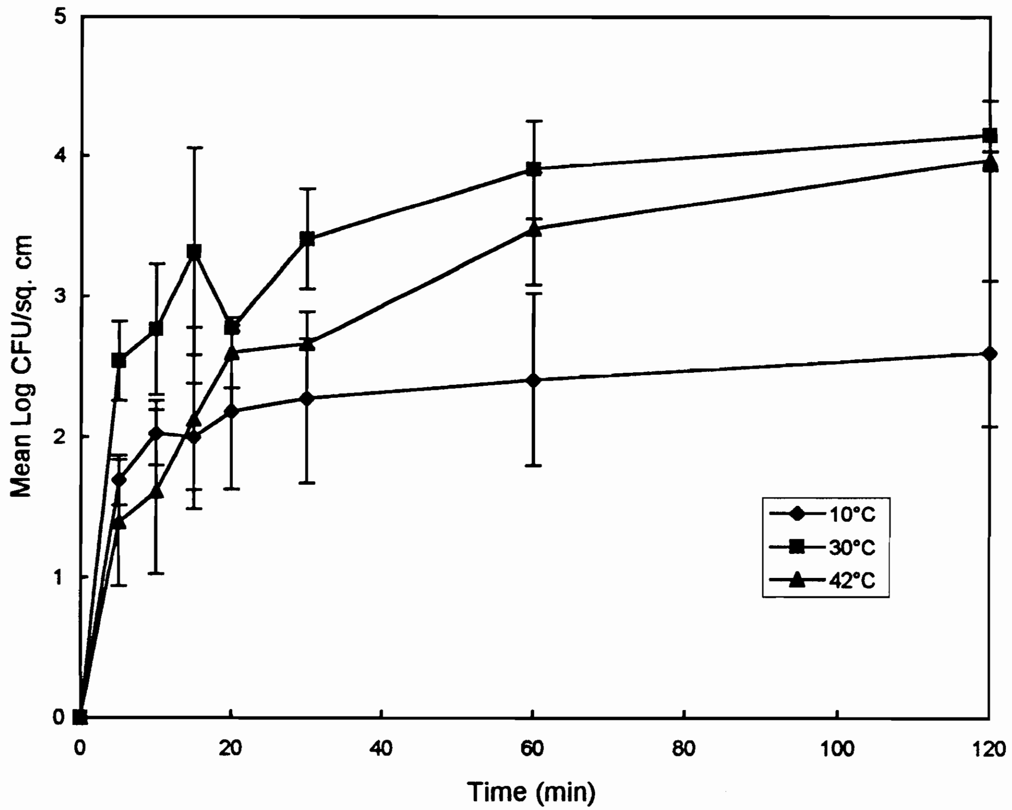

Figure 4. Attachment profile for Listeria monocytogenes *Scott A grown to mid-log at 10°, 30°, and 42°C in TSB-YE (pH 7.0) and exposed to Buna-N rubber at 30°C in PB (pH 7.0) for 120 min.*

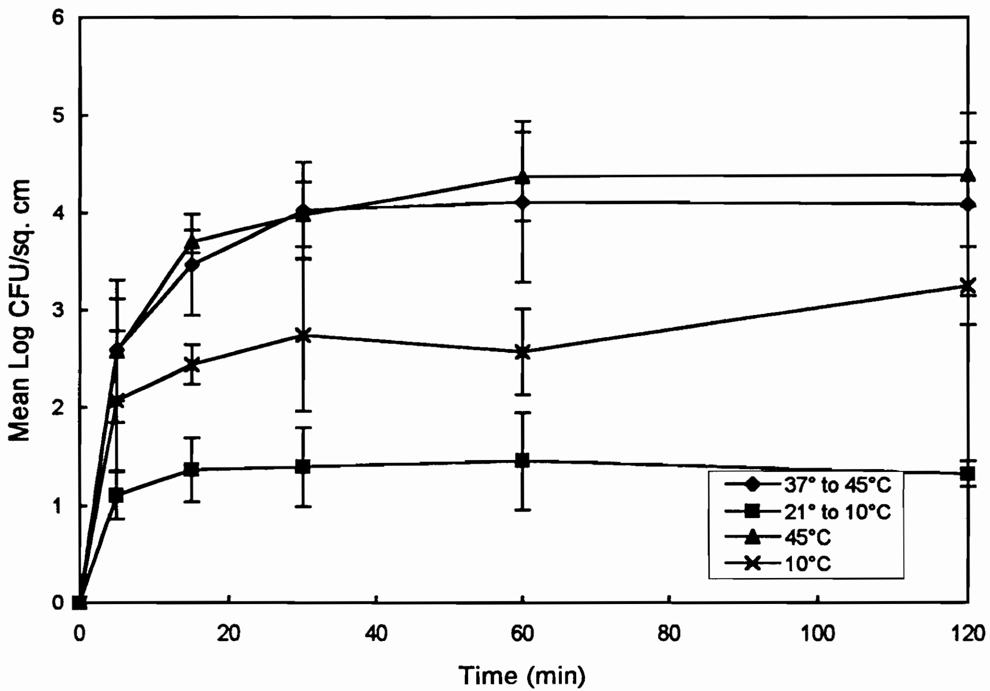

Figure 5. Attachment profile for Listeria monocytogenes *Scott A grown to mid-log at 30°C in TSB-YE (pH 7.0), held at either 37°or 21°C for 15 Min in PB (pH 7.0) and exposed to Buna-N rubber at 45° and 10°C in PB (pH 7.0), respectively, for 120 min.*

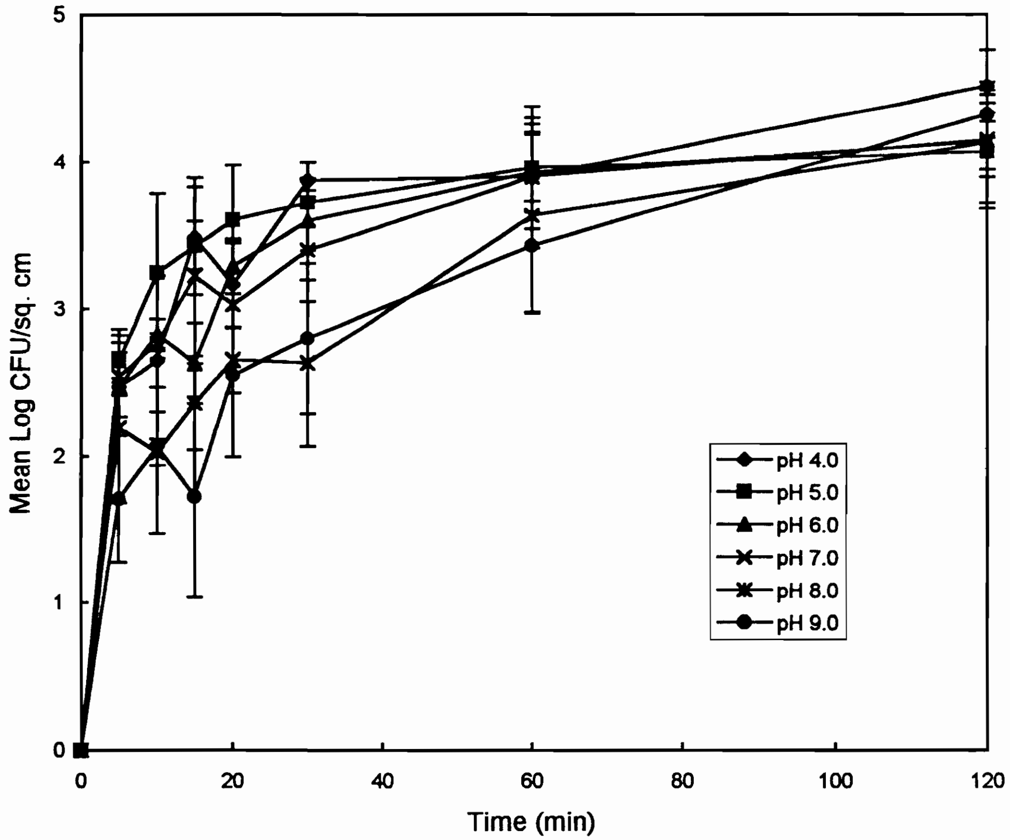

Figure 6. Attachment profile for Listeria monocytogenes *Scott A grown to mid-log at 30°C in TSB-YE (pH 7.0) and exposed to Buna-N rubber in PB adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 for 120 min.*

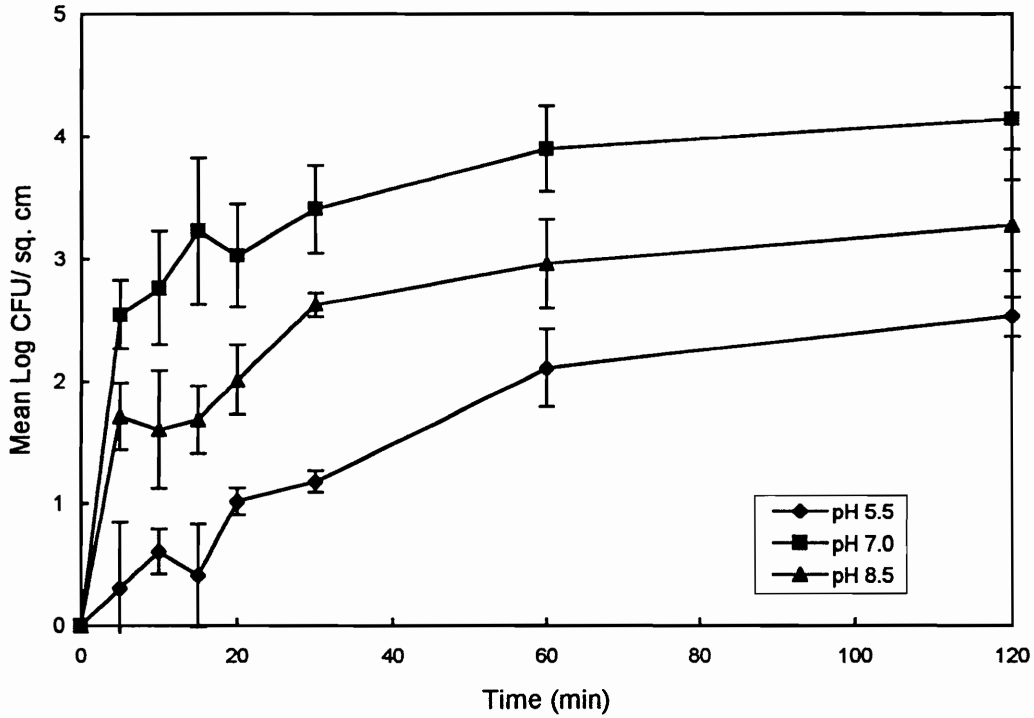

Figure 7. Attachment profile for Listeria monocytogenes *Scott A grown to mid-log in pH 5.5, 7.0, and 8.5 TSB-YE at 30°C and exposed to Buna-N rubber at 30°C in PB (pH 7.0) for 120 min.*

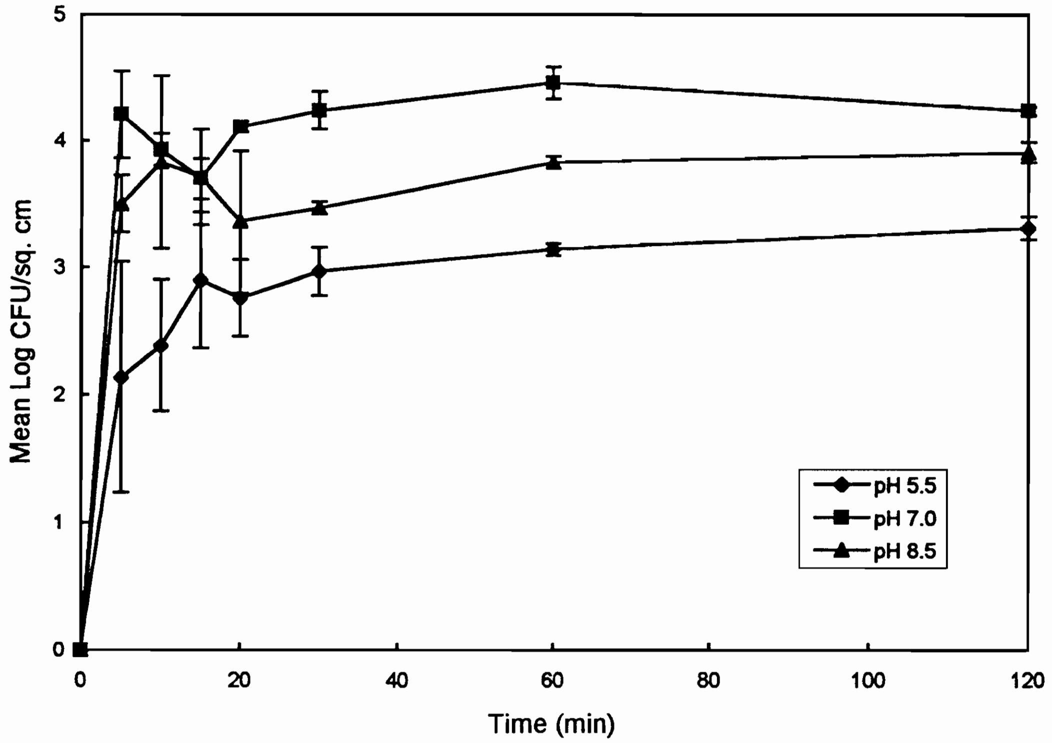

Figure 8. Attachment profile for Listeria monocytogenes *Scott A grown to mid-log in pH 5.5, 7.0, and 8.5 TSB-YE at 30°C and exposed to stainless steel at 30°C in PB (pH 7.0) for 120 min.*

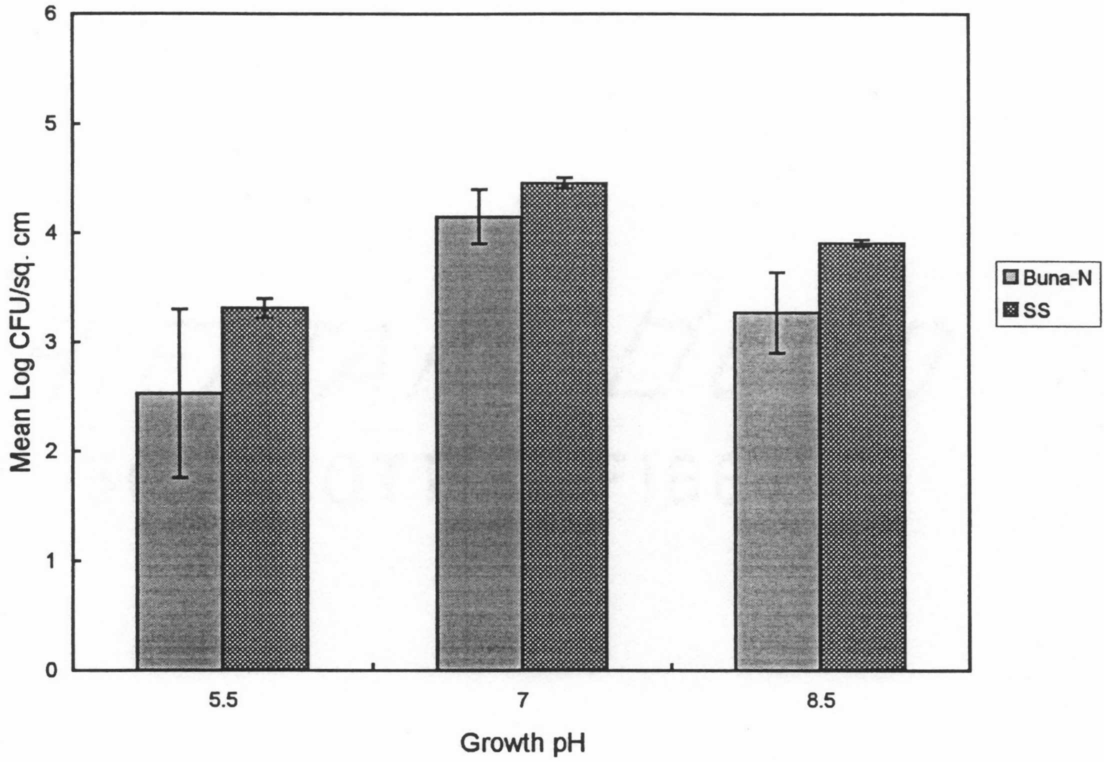

Figure 9. Effect of growth pH on maximum number of Listeria monocytogenes *Scott A detected on Buna-N rubber and stainless steel surfaces after 120 min exposure in PB at 30°C.*

IV. INFLUENCE OF ENVIRONMENTAL STRESS ON KINETICS OF ATTACHMENT OF *LISTERIA MONOCYTOGENES* SCOTT A TO BUNA-N RUBBER AND STAINLESS STEEL

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ABSTRACT

Attachment and detachment of *Listeria monocytogenes* Scott A to Buna-N rubber and stainless steel under varying conditions of temperature and pH were investigated in laboratory model systems. Numbers of attached cells increased with increasing attachment temperature (10° to 45°C) and time (up to 120 min) for both test surfaces. Compared to Buna-N rubber, the rate of attachment to stainless steel was markedly more rapid for all temperature and pH conditions studied and could not be calculated. Rate of attachment to Buna-N rubber was found to be significantly lower when cells were attached at 10°C. Growth temperature did not significantly affect rates of adhesion to Buna-N rubber. Altering the medium pH during attachment between 4 to 9 demonstrated that rates of adhesion were slower under alkaline conditions. Growth pH was also found to significantly affect rates of attachment to Buna-N rubber. Detachment of cells adhered to Buna-N rubber was significantly affected by growth temperature, but not growth pH. Significant differences in detachment were also found between Buna-N rubber and stainless steel, inferring stronger attachment to Buna-N rubber. Cell surface hydrophobicity was found to be affected by both growth temperature and growth pH. However, changes in hydrophobicity could not be correlated to differences in rates of attachment. Addition of 0.01% trypsin to the attachment medium during cell exposure to either test surface resulted in a 99.9% reduction in the adhered cell population when compared to controls. This would suggest that proteins play a role in the initial attachment process of *L. monocytogenes*.

INTRODUCTION

Over the past decade, *Listeria monocytogenes* has been regarded as one of the leading causes of foodborne illness. The organism has been found in a wide range of food products including dairy, meat, poultry, seafood as well as fruits and vegetables (*30*). Its high mortality rate, wide occurrence in nature and ability to grow at refrigeration temperatures has prompted regulatory agencies in some countries to implement a zero tolerance of *L. monocytogenes* in ready-to-eat foods. For microorganisms in which a zero tolerance is in effect, the presence of attached cells may be as significant a threat to the production of safe food as well developed biofilms (*17*). The ability of *L. monocytogenes* to attach to various surfaces has been well documented (*4,12,14,19,21,25*). The presence of *L. monocytogenes* on food contact surfaces represents a source of potential contamination. From the moment of initial attachment, detachment of microbial cells and related biofilm material from surfaces occurs (*7*). The ease with which microorganisms shed from surfaces contributes to their ability to spread and contaminate food product items in their vicinity (*9*).

Microorganisms in the environment behave differently from those grown under optimal conditions in laboratory media. Recently, numerous studies investigating the effects of environmental stress on microorganisms have been published (*5,11,18,23,24,29,32,33*). Responses to environmental stress include a decrease in cell size, altered growth rate, changes in the cell surface properties such as degree of

hydrophobicity and an increased resistance to severe stress or stresses due to altered protein synthesis. Leyer and Johnson (*23*) observed the synthesis of specific outer membrane proteins and increased cell surface hydrophobicity in acid adapted cells of *Salmonella typhimurium*. These researchers suggested that acid stress could affect the ability of this microorganism to attach to hydrophobic surfaces. Herald and Zottola (*15*) first studied the attachment of *L. monocytogenes* to a food contact surface using SEM. Other researchers have used similar SEM techniques to describe *L. monocytogenes* attachment to various food contact surfaces such as, stainless steel, polypropylene, and rubber (*25*). The environmental conditions which affect the rate of adhesion of *L. monocytogenes* to food contact surfaces, however, are not well understood or defined.

Studies from other scientific disciplines have reported Langmuir isotherm-type curves for attachment of microorganisms to surfaces (*6,31*) . Although Langmuir's theory of gas adsorption has typically been applied to ideal particles, the Langmuir equation has been useful in describing the kinetics of attachment of a number of microorganisms. In a previous study, we reported on the attachment of *L. monocytogenes* to stainless steel and Buna-N rubber under different environmental conditions (*38*). In this study we further investigate how temperature and pH affects attachment to food contact surfaces by examining rates of attachment and ease of detachment.

MATERIALS AND METHODS

Test organism and culture maintenance

L. monocytogenes strain Scott A was obtained from the culture collection of the Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg VA. The culture was maintained on trypticase soy agar (TSA, Difco, Detroit, MI) supplemented with 0.6% yeast extract (YE, Difco) slants (TSA-YE) held at 4 °C. Prior to use, the culture was subcultured on 2 consecutive days in trypticase soy broth (TSB, Difco) supplemented with 0.6% yeast extract (Difco) (TSB-YE) and incubated at 37 °C.

Test surfaces

Buna-N rubber, obtained from M. G. Newell Greensboro, NC, and stainless steel (type 304, #4 finish), were used for attachment of *L. monocytogenes*. The test surfaces were cut into 2 x 5 cm coupons (ca. 2 mm in thickness), washed by soaking in warm alkaline cleaner (Klenzade, Division of Ecolab Inc., St. Paul, MN) overnight and rinsed repeatedly with distilled water. The surfaces were sterilized by autoclaving for 15 min at 121°C .

Attached cell development

One ml of a 18-24 h TSB-YE culture was used to inoculate 250 ml of TSB-YE pre-tempered to the growth temperature. Cells were incubated at the growth temperature with shaking (100 rpm) and grown to mid-log phase based on previous growth curve studies. Mid-log phase cells were harvested by centrifugation, washed and resuspended in an equal volume of sterile phosphate buffer (0.02 M Potassium Phosphate Buffer, pH 7.0) (PB). One ml aliquots of the washed cell suspension were used to inoculate multiple 250 ml screw cap flasks containing 99 ml of PB pre-tempered to the attachment temperature to give a target level of 1 x 10^7 CFU/ml. After incubation for 15 min with shaking (100 rpm) at the attachment temperature, 1 sterile Buna-N rubber or stainless steel coupon pre-tempered to the attachment temperature was added to each flask. Incubation with shaking was continued at the test temperature. Coupons for each test surfaces were removed after 0, 5, 10, 15, 20, 30, 60, and 120 min of exposure. Test surfaces were prepared for enumeration and attached cells were quantitated as described below.

Enumeration of attached cells

To enumerate attached cell populations the test surfaces were first rinsed in 20 ml volumes of the sterile PB. This rinse step was repeated 4 times to remove any reversibly attached cells. Viable counts on the rinse buffer established that less than 1 CFU per ml was detected in the fifth rinse. After the last rinse, the buffer was aspirated and the side of the surface which had been positioned upward in the flask during attachment was swabbed with a calcium alginate swab. The swab was transferred to 10 ml of sterile PB and the

sample serially diluted in 0.1% peptone. Attached cells were enumerated on TSA-YE spread plates incubated at 37°C for 48 h. Duplicate surfaces were enumerated at each attachment period and the average number of cells was calculated as CFU per cm^2 test surface. Swabbed surfaces were pour plated with TSA-YE and incubated at 37°C for 48 h to ensure that all attached cells had been removed.

Effect of temperature on attachment

Attachment profiles developed in previous experiments (*38*) investigating the effects of temperature were used to calculate rates of adhesion. Rates of adhesion were determined for cells grown at either 10°, 30° or 42°C and attached at 30°C to Buna-N rubber and cells grown at 30°C and attached at either 10°, 30° or 45°C to stainless steel and Buna-N rubber.

Effect of pH on attachment

Attachment profiles developed in previous experiments (*38*) investigating the effects of pH were used to calculate rates of adhesion. Rates of adhesion were determine for cells grown at either pH 5.5, 7.0 or 8.5 and attached at pH 7.0 to Buna-N rubber and stainless steel as well as for cells grown at pH 7.0 and attached at either pH 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0 to Buna-N rubber.

Salt Aggregation Test

The effect of growth temperature on the cell surface hydrophobicity was determined using the salt aggregation test (SAT) as described by Rozgonyi et al. (*35*) and Mafu et al. (*26*). One ml of an 18-24 h culture was used to inoculate 250 ml of TSB-YE pre-tempered to either 10°, 30° or 42°C. Cells were grown to mid-log with shaking (100 rpm). For each growth temperature, 1 ml was centrifuged and resuspended in an equal volume of 0.02 M sodium phosphate buffer containing 0.85% NaCl (pH 6.8). Twenty-five μ l was placed on a glass depression slide and mixed with 40 μl of 2.0, 3.0, and 4.0 M ammonium sulfate in 0.02 M sodium phosphate buffer. The bacterium-salt solution mixture was gently rotated for 15 min at room temperature using a Tekmar shaker. The lowest concentration of ammonium sulfate resulting in aggregation visible with the naked eye under white-light illumination was scored as the positive SAT hydrophobicity value. Hydrophobicity measurements were repeated 4 times for each growth temperature. The effect of growth pH on the hydrophobicity of *L. monocytogenes* was similarly determined on mid-log phase cells grown at 30°C in TSB-YE adjusted to either pH 5.5, 7.0 or 8.5.

Effect of trypsin on attachment

Trypsin IX (EC 3.4.21.4) was obtained from Sigma Chemical Co., St. Louis, MO. A stock solution of 1.0 % (w/v) trypsin was prepared fresh each day of use by dissolving in distilled water and filter sterilizing with a 0.45 μm Acrodisc (Gelman Sciences, Ann Arbor, MI).

Cells were grown to mid-log phase at 30°C and the test suspension was prepared as previously described. One ml of the stock trypsin solution was added to 99 ml of the test suspension to give a final concentration of 0.01% (w/v) trypsin. After 15 min at 30°C with shaking, the test surfaces were added. After the pre-determined exposure times, surfaces were removed and both planktonic and attached cells enumerated. Duplicate trials were performed for each test surface.

Rate of attachment

Rates of attachment for *L. monocytogenes* under selected temperature and pH conditions were calculated as follows for the first 60 min of cell exposure to the test surface. Based on the Langmuir analysis of molecular adsorption, the concentration of irreversibly adsorbed cells as a function of time, n(t) may be written as

$$\frac{d\theta}{dt} = k \cdot c(t) \cdot N[1 - \theta(t)] \qquad (1)$$

where N is the number of surface sites and is dependent on the surface area of the test surface. C(t) is the concentration of the cells at time t. $\theta(t)$ is the coverage of the surface as a function of time and is given by

$$\theta = \frac{n_0 - n(t)}{n_0 - n_f} \qquad (2)$$

where n_0 and n_f are the initial and final concentrations of cells in suspension, respectively. Integration of equation 1 yields

$$\ln (1 - \theta) = -k \cdot c(t) \cdot N(t) \qquad (3)$$

A plot of ln (1 - θ) versus t should give a straight line from which k can be determined when the number of sites and cell concentration are held constant (e.g. slope = $-k \cdot C(t) \cdot N$). When straight line plots are not obtained, k is implied to be dependent on coverage (*20*).

Detachment assays

The mathematical expression used by Eginton et al. (*9*) to describe the ease of removal of *Pseudomonas aureginosa*, *Staphylococcus epidermidis* and *Escherichia coli* from various food processing surfaces, was used in this study to investigate the removal of *L. monocytogenes* attached to the test surfaces under different temperature and pH conditions. To determine the effect of growth temperature on the removal of *L. monocytogenes* from Buna-N rubber, cells were grown to mid-log phase at either 10°, 30° or 42 °C in TSB-YE and the test suspension prepared as previously described. Buna-N rubber surfaces were exposed to the test cell suspension for 60 min and rinsed prior to enumeration of attached cells. The surfaces were then gently placed on a pre-dried TSA-YE plates with the side positioned upward in the test suspensions in contact with the agar

surfaces. After 1 min, the surfaces were removed and similarly placed on fresh pre-dried TSA-YE plates. This step was repeated for at least 20 successive plates. To facilitate spread plating, 0.1 ml of 0.1% peptone was added to the agar surfaces. For the last transfer, the surfaces were placed face up in a petri dish and pour plated with TSA-YE. Colonies remaining on the surface were visible after 48 h incubation at 37°C. To compare differences in ease of removal between surface types, stainless steel coupons were exposed to the test cell suspension of *L. monocytogenes* grown to mid-log at 30°C and analyzed as described above.

Cells were grown at 30°C in TSB-YE adjusted to either pH 5.5, 7.0 or 8.5 to determine the effect of growth pH on the removal of *L. monocytogenes* from Buna-N rubber. The test surface was exposed to the cell suspension (PBS, pH 7.0) for 60 min, surfaces rinsed and attached cells enumerated by successive plating on TSA-YE as described for the temperature trials. All surfaces were evaluated in triplicate for both temperature and pH experiments.

The mean number of CFU/plate was plotted against the plate succession number and lines of best fit were determined by linear regression. The data was fitted to the following equation

$$CFU = A\ 10^{-kN} \qquad (4)$$

where CFU is the number of colonies on any given TSA-YE plate, A is a constant, N is the plate succession number and k is a reduction exponent.

Statistical analysis of data

All attachment experiments examined in this study for adhesion rate determinations were performed in triplicate utilizing replicate coupons. Data were analyzed using two-way analysis of variance (Excel software, Microsoft Corp., Redmond, WA) for all experimental conditions and surface combinations. All detachment experiments were repeated at least 3 times and mean reduction exponents were analyzed by one-way analysis of variance. Means for both attachment and detachment experiments were separated using Duncan's multiple range test employing an α level of 0.05.

RESULTS

Effect of temperature and pH on rate of attachment

Rates of attachment (k, min^{-1}) for the growth and attachment temperature combinations were determined over the first 60 min of cell exposure to Buna-N rubber (Table 1). No significant difference ($P > 0.01$) was found in the rates of attachment determined at the three growth temperatures; 10°, 30°, and 42°C. The attachment rate of cells grown to mid-log at 30°C and attached at 10°C to Buna-N rubber were significantly lower ($P < 0.001$) than the rates obtained for 30°C grown cells attached at 30° or 45°C.

The effect of growth and attachment pH on the rate of adherence by *L. monocytogenes* to Buna-N was determined (Table 2). Cells grown to mid-log at pH 7.0 had a significantly ($P < 0.001$) higher rate of attachment when compared to cells grown at pH 5.5, but not when compared to cells grown at pH 8.5. Rates determined for the different pH values, indicated slower attachment occurred at alkaline pH. At pH 9, attachment occurred at a rate significantly slower ($P < 0.001$) than rates obtained at pH 7 and lower. Attachment rates obtained under acidic conditions were not significantly different from attachment rates obtained under neutral conditions.

Compared to Buna-N rubber, attachment of *L. monocytogenes* to stainless steel was noticeably more rapid for all temperature and pH combinations investigated. However, attachment rates to stainless steel could not be determined, since after 5 min of exposure to the test surface, adhered cell concentrations were already at or near the maximum level of attachment observed during the 120 min sampling period.

Effect of temperature and pH on hydrophobicity

The results of the SAT for *L. monocytogenes* grown at different temperatures and pH levels are presented in Figure 1. Cells grown at 30°C in TSB-YE (pH 7.0) were more hydrophilic (i.e. aggregating at 4 M ammonium sulfate) than those cells grown at either 10° or 42°C in the same growth medium. When cells were grown in TSB-YE adjusted to pH 8.5, the cell surface was found to be more hydrophobic (i.e. aggregating at 2 M ammonium sulfate) than the surfaces of those cells grown in TSB-YE adjusted to either pH 5.5 or 8.5.

Effect of trypsin on attachment

The effect of trypsin on attachment of *L. monocytogenes* to Buna-N rubber and stainless steel was determined (Figure 2). Cells exposed to the Buna-N rubber at 30°C in the presence of 0.01% trypsin reached a maximum attachment concentration of < 10 CFU/cm^2 after 120 min of surface exposure. No attachment was observed during the first 30 min of exposure. Low concentrations of adhered cells were also observed on stainless steel when cells were exposed to this surface in the presence of trypsin. Maximum concentrations of attachment obtained on stainless steel approached 10 CFU/cm^2. Planktonic cell concentrations were measured during the exposure period to determine if the enzyme had affected cell viability. Levels of viable planktonic cells remained unchanged throughout the 120 min exposure period. Compared to levels of attachment

observed in the absence of trypsin, adhered cell populations over the 120 min exposure were reduced by 99.9% on both test surfaces.

Effect of growth temperature and pH on detachment

Using the successive blotting/transfer plate method of Eginton et al. (*9*), the detachment of *L. monocytogenes* adhered to test surfaces under selected environmental conditions was investigated. Results of experiments performed to characterize the effect of growth temperature (i.e., 10°, 30°, and 42°C) on detachment of adhered cells from Buna-N rubber are graphically illustrated in Figure 3. Similar experiments were performed for cells grown at 30°C and adhered to stainless steel (data not shown). The influence of growth medium pH (i.e., 5.5, 7.0, and 8.5) on detachment of cells adhered to Buna-N rubber is shown in Figure 4. For both growth parameters investigated, it was found that as the plate succession number increased, the mean CFU per plate decreased exponentially. Regression analyses were performed and lines of best fit determined. Details of the regression analysis of cell detachment from the two test surfaces are summarized in Table 3. The reduction exponents (e.g., k) for the lines of best fit differed significantly ($P < 0.01$) between adhered cells grown at 10°C and cells grown at 30°C, for Buna-N rubber. However, reduction exponents determined for cells grown at different pH values prior to attachment to Buna-N rubber were found not to differ significantly. The k values determined for cells grown at 30°C at pH 7.0 and adhered to both Buna-N rubber and stainless steel were found to differ significantly ($P < 0.01$).

DISCUSSION

L. monocytogenes has been quantitatively shown to attach to Buna-N rubber and stainless steel within short contact times (*38*). The ability of *L. monocytogenes* to attach to Buna-N rubber and stainless steel has also been previously reported by several workers using SEM techniques (*15,25*). Buna-N rubber and stainless steel were used in this study because of their widespread use in the food industry and because they differ in their surface properties. Buna-N rubber is hydrophobic and has a lower surface free energy than stainless steel. Stainless steel is hydrophilic, although it is thought to possess hydrophobic regions. Substratum properties for food contact surfaces have also been characterized (*26*). Studies investigating the affect of surface properties (e.g., hydrophobicity, surface charge, surface free energy, and extracellular polymer production) for both substratum and bacterium, on attachment have been reported (*2,10,13,26,36*).

Environmental conditions are known to affect cell surface characteristics (*3,23*). Growth rate as well as growth phase have been shown to influence the regulatory mechanisms which determine cell surface properties (*13*). Very little work has been directed toward identifying environmental variables which influence the adhesion rates of microorganisms to food contact surfaces using defined biological systems. Rutter and Vincent (*36*) suggested the need for further investigations on the relative importance of extracellular polymers, specific and nonspecific binding sites, metabolic rate, motility,

pH , temperature, etc. on microbial attachment. Attempts in other scientific disciplines to describe the microbial adhesion process have largely been in terms of colloidal chemistry or surface free energy. The theory of surface free energy predicts adhesion will occur when the process results in a decrease in free energy. However, Mafu et al. (*26*), did not find a correlation between the surface energy of a substratum and the ability of *L. monocytogenes* to attach. Several researchers have noted that the kinetics of adhesion to different substrata leads to Langmuir isotherm-type curves (*6,31*). Attachment profiles for *L. monocytogenes* to Buna-N rubber and stainless steel exhibited typical Langmuir adsorption curves (*38*). Therefore, the Langmuir equation for irreversible adsorption was used to determine the rates of attachment to Buna-N rubber for the different environmental conditions.

We found that the temperature at the time of attachment affected the ability of *L. monocytogenes* to attach to both Buna-N rubber and stainless steel (Table 1). For Buna-N rubber, cells grown at 30°C adhered at a faster rate when attachment occurred at 45°C. Attachment occurred at a significantly slower rate at 10°C when compared to the higher temperatures investigated. Since attachment was greater with an increase in temperature, regardless of the surface, the higher temperatures may have lowered the energy of activation of chemical reactions at the cell-substratum interface which are involved with the attachment process.

Growth temperature prior to attachment did not affect the rate of adhesion to Buna-N rubber (Table 1). *L. monocytogenes* possess multiple nonpolar flagella at

temperatures less than 30°C (*15*). Surface structures such as flagella and fimbriae are thought to play a role in attachment (*10*). Growth at 10°C did not significantly influence the rate of attachment. The differences in rates of adhesion noted in this study cannot be attributed to growth phase, since all cells were grown to mid-log phase prior to attachment.

We applied the equation of Arrhenius to further investigate how temperature affects the rate of bacterial attachment within the temperature range studied. The Arrhenius equation which has been used to describe how temperature affects the rate of chemical reactions is given below:

$$v = S\,e^{-\Delta E/RT} \qquad (5)$$

Where v is the velocity of the reaction, S is a constant, ΔE is the activation energy of the reaction, R is the gas constant and T is the temperature in K. In its logarithmic form the equation becomes

$$\log v = (-\Delta E/2.3\,R)\,(1/T) + S \qquad (6)$$

The logarithm of the velocity of a chemical reaction is a linear function of the reciprocal of absolute temperature: the line has a negative slope ($-\Delta E/2.3R$) from which the value of the activation energy can be calculated. If an Arrhenius plot is made for bacterial rate of

attachment (k), rather than for the chemical reaction rate, a similar response is observed (Figure 5). This plot demonstrates that for the temperature range studied, normal chemical kinetics seem to apply. To our knowledge, this is the first time that a relationship between temperature and rate of attachment for *L. monocytogenes* to a food contact surface has been reported. From this plot, the E_a for attachment of *L. monocytogenes* to Buna-N rubber can be calculated for a given temperature. The magnitude of the E_a indicates the temperature dependence of the reaction. The determination of the E_a may be useful in comparing the temperature dependence of *L. monocytogenes* attachment to various food contact surfaces under different environmental conditions.

Initial rates of attachment determined on Buna-N rubber did not differ between pH 4.0 to pH 7.0 (Table 2). Alkaline pH levels (i.e. 8.0 and 9.0) however, resulted in a decrease in the rate of attachment. Structures on the cell surface possessing charged groups such as lipopolysaccharides and proteins have been shown to be affected by changes in pH. Sign and amplitude of the cell surface potential is determined by the pH in the environment (*28*). Several researchers have confirmed the production of a polysaccharide by *L. monocytogenes* (*15,25,36*). A number of bacteria produce extracellular polymers which are thought to promote irreversible attachment. These polymers have been identified as acidic polysaccharides (*39*). As the pH increases, there is an increase in the overall anionic charge of these polymer molecules resulting from the dissociation of acidic groups. Lewis et al., (*22*) suggested that since the majority of

surfaces in contact with aqueous solutions are negatively charged, electrostatic repulsion between the bacterial surface and solid substratum would be greater at a more alkaline pH.

The pH during growth prior to attachment was shown to affect the rate of attachment observed on both Buna-N rubber and stainless steel (Table 2). Cells grown at pH 7.0 attached to Buna-N rubber at significantly higher rates when compared to the other growth pH values. The affect of growth pH was greater on Buna-N rubber than on stainless steel. Although the growth pH was also shown to alter the cell surface hydrophobicity (Figure 1), the difference in hydrophobicity could not be correlated to the observed attachment of *L. monocytogenes* to the test surfaces.

The surface free energy of *L. monocytogenes* is approximately 66 mJ $\cdot$ m^{-2} (*1,26*). Absolom et al., (*1*) classified a microorganism as hydrophilic when its surface free energy is within the range of 65 to 69 mJ $\cdot$ m^{-2}. The hydrophobicity of various strains of *L. monocytogenes* Scott A as determined by hydrophobic interaction chromatography (HIC) and SAT has been previously reported (*26*). Low pH or high ionic strengths were required for the expression of hydrophobicity. *L. monocytogenes* was found to bind to the octyl-sepharose column only when the pH neared the isoelectric point of the microorganism (i.e. pH range 2 to 3.5). Using the SAT, these same researchers also reported aggregation of cells with 3 and 4 M ammonium sulfate. Our results further confirm the low hydrophobic nature of this microorganism. In addition, it was found that altering the pH or temperature of the growth environment resulted in changes in the cell surface hydrophobicity (Figure 1).

In this study, *L. monocytogenes* was found to attach at a faster rate to stainless steel than to Buna-N rubber, irrespective of the environmental conditions. These findings are consistent with others who have used SEM techniques to demonstrate greater attachment to stainless steel when compared to Buna-N rubber (*15,25*). The surface free energies based on polar and dispersion components would thermodynamically predict that adhesion of *L. monocytogenes* would be more energetically favorable as the surface free energy of the solid substratum decreases. Rubber surfaces have a lower surface free energy than stainless steel and adhesion would be expected to be more favorable to this surface. However, factors other than cell surface hydrophobicity such as surface charges and the presence of an exopolymer, may also be important in the adhesion process to stainless steel, glass, polypropylene and rubber surfaces (*26*).

Marshall et al. (*27*) described the attachment process as a two stage process (i.e. reversible and irreversible). The bacterium comes in contact with the surface and is weakly held in place by electrostatic and Van der Waals Forces. The second or irreversible attachment involves physical attachment of the cell to the surface by extracellular polymeric substances produced by the cell. The role of extracellular polymers in attachment is, to date, unclear. Using carbohydrate disrupting compounds, Herald and Zottola (*16*) demonstrated a decrease in adherence of *P. fragi* to stainless steel. Other reports indicate that extracellular material is not required for attachment but may help stabilize biofilms (*2,8*). Paul and Jeffrey (*34*) proposed that attachment of *Vibrio proteolytica* to hydrophilic and hydrophobic surfaces occurs by different mechanisms.

Using proteolytic enzymes, these researchers found greater than 97% inhibition of attachment to hydrophobic surfaces. These same enzymes however did not affect attachment to hydrophilic surfaces. In this study, attachment of *L. monocytogenes* to Buna-N rubber and stainless steel in the presence of trypsin was reduced by 99.9% on both test surfaces over the 120 min exposure. The reduction in attachment observed in the presence of a proteolytic enzyme would suggest that proteins rather than polysaccharides play a role in the initial attachment process.

Another approach for studying attachment has been the investigation the detachment of microorganisms from surfaces. Eginton et al., (*9*) investigated the ease of removal of several microorganisms from surfaces possessing different surface properties. They inferred that the ease of removal could be related to the strength of attachment to that surface by comparison of reduction exponents. It would be beneficial to know what environmental conditions, if any, favor detachment. Hood and Zottola (*17*) have introduced the term biotransfer potential as any microorganism associated with a surface that could eventually lead to contamination of food. In this study, after an 1 h contact time in PBS (pH 7.0), reduction coefficients calculated from successive plating of the surface on TSA-YE did not differ for adhered cells grown at the 3 different pH levels prior to attachment. As previously mentioned, growth pH was found to affect the rate of attachment and cell surface hydrophobicity. However, changes in the cell surface hydrophobicity were not found to influence the apparent strength of attachment. Although attachment of *L. monocytogenes* to stainless steel occurred at a faster rate and to a greater

extent, comparison of reduction exponents determined for cells grown at 30°C and attached to either surface at 30°C indicated that the strength of attachment was greater on Buna-N rubber.

In summary we have shown that exposure of *L. monocytogenes* to environmental stresses, such as pH and temperature, can significantly affect the rate and strength of attachment of this pathogen to common food contact surfaces. Inhibition of attachment in the presence of a proteoplytic enzyme suggests proteins are involved in the initial attachment process of *L. monocytogenes*. Given that extreme environmental conditions are known to dramatically alter protein synthesis, further investigations into the relationship between stress related proteins and the ability of pathogenic microorganisms to adhere to surfaces is warranted. Such studies could provide a better understanding of the attachment mechanisms to food contact surfaces and perhaps lead to better methods of control of *L monocytogenes* in the food processing environment.

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TABLE 1. *Effect of temperature on the rate of attachment by* Listeria monocytogenes *Scott A to Buna-N rubber in PB (pH 7.0).*[A, B]

	Growth Temperature (°C)	Attachment Temperature (°C)	k (min^{-1}) ± s.d
I.	10	30	0.0323[a] ± 0.0035
	30	30	0.0469[a] ± 0.0156
	42	30	0.0477[a] ± 0.0140
II.	30	10	0.0229[a] ± 0.0072
	30	30	0.0469[b] ± 0.0156
	30	45	0.0577[b] ± 0.0150

A - Data represent mean attachment rates obtained from 3 independent trials. k values were determined during the first 60 min of surface exposure at each temperature combination.

B - Means within the same data set having different letters are significantly different at $P < 0.001$.

TABLE 2. *Effect of pH on the rate of attachment by* Listeria monocytogenes *Scott A to Buna-N rubber in PB at 30°C.*[A,B]

	Growth pH	Attachment pH	k (min^{-1}) ± s.d
I.	5.5	7.0	0.0240[a] ± 0.0009
	7.0	7.0	0.0469[b] ± 0.0156
	8.5	7.0	0.0378[a,b] ± 0.0084
II.	7.0	4.0	0.0535[a] ± 0.0242
	7.0	5.0	0.0592[a] ± 0.0130
	7.0	6.0	0.0491[a] ± 0.0261
	7.0	7.0	0.0469[a,b] ± 0.0156
	7.0	8.0	0.0346[b,c] ± 0.0114
	7.0	9.0	0.0265[c] ± 0.0071

A - Data represent mean attachment rates obtained from 3 independent trials. k values were determined during the first 60 min of surface exposure at each pH combination.

B - Means within the same data set having different letters are significantly different at $P < 0.001$.

TABLE 3. *Regression analysis of adhered* Listeria monocytogenes *Scott A removed from Buna-N rubber and stainless steel by successive blotting on TSA-YE plates. CFU =* $A \cdot 10^{-kN}$*, where CFU is the number of colonies transferred, k is the removal exponent, A the intercept and N the plate succession number.*[A,B]

Test Surface	Growth Temperature (°C)	Growth pH	A	k	r^2
I. Buna-N rubber	10	7.0	2.85	-0.13[a]	0.92
	30	7.0	2.75	-0.08[b]	0.96
	42	7.0	2.63	-0.10[a,b]	0.91
II. Buna-N rubber	30	5.5	2.54	-0.10	0.95
	30	7.0	3.17	-0.08	0.96
	30	8.5	2.92	-0.09	0.93
III. Buna-N rubber	30	7.0	3.17	-0.08[a]	0.96
Stainless steel	30	7.0	5.52	-0.20[b]	0.89

A - Data represent means obtained from 3 independent trials.

B - k values within the same data set having different letters are significantly different at the $P < 0.01$ level.

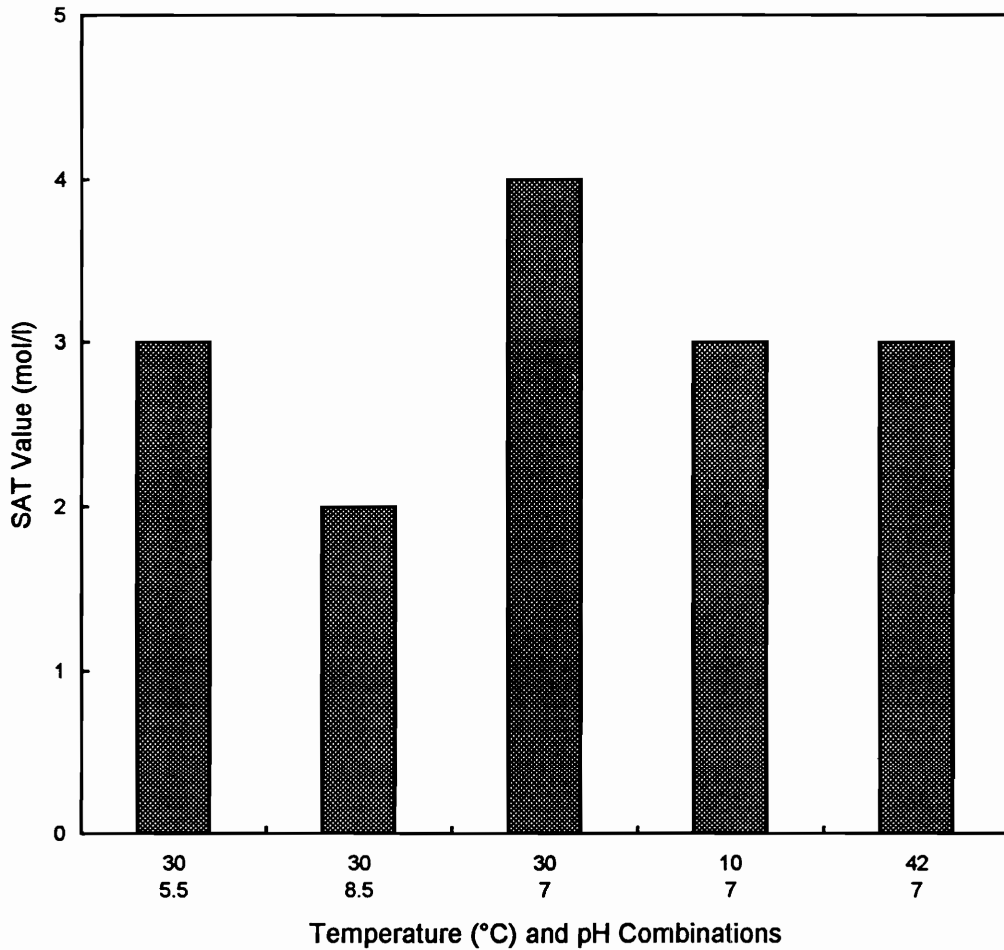

Figure 1. SAT values as a measure of hydrophobicity for L. monocytogenes *Scott A grown in TSB-YE at various pH and temperature combinations.*

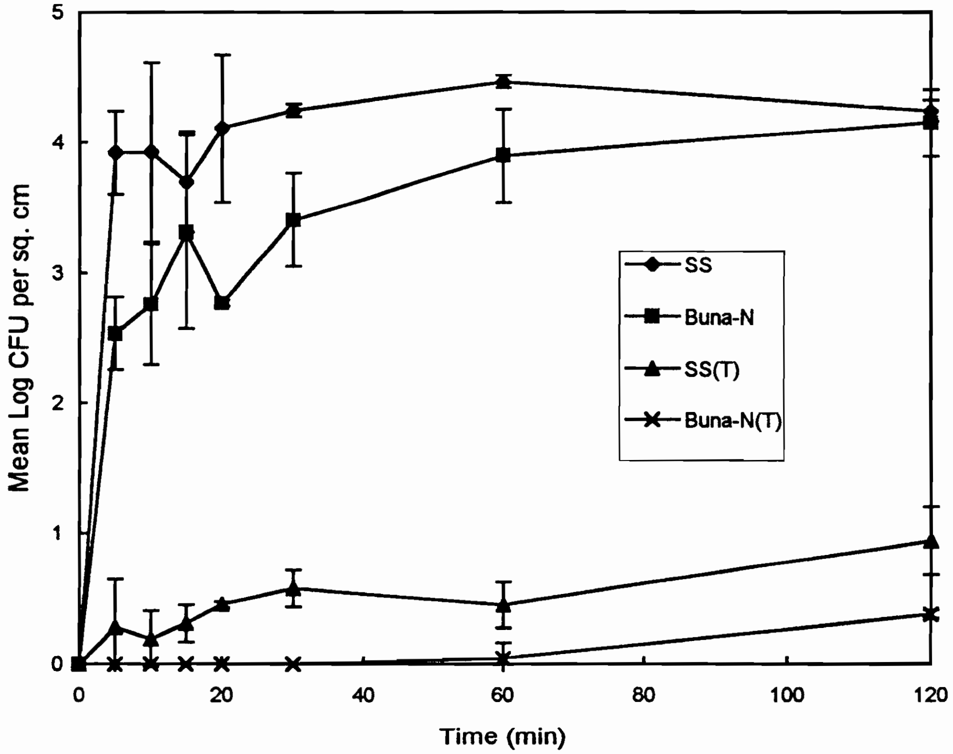

Figure 2 . Listeria monocytogenes *Scott A grown to mid-log at 30°C and attached to Buna-N rubber (Buna-N) and stainless steel (SS) at 30°C in PB (pH 7.0) with or without 0.01% trypsin (T).*

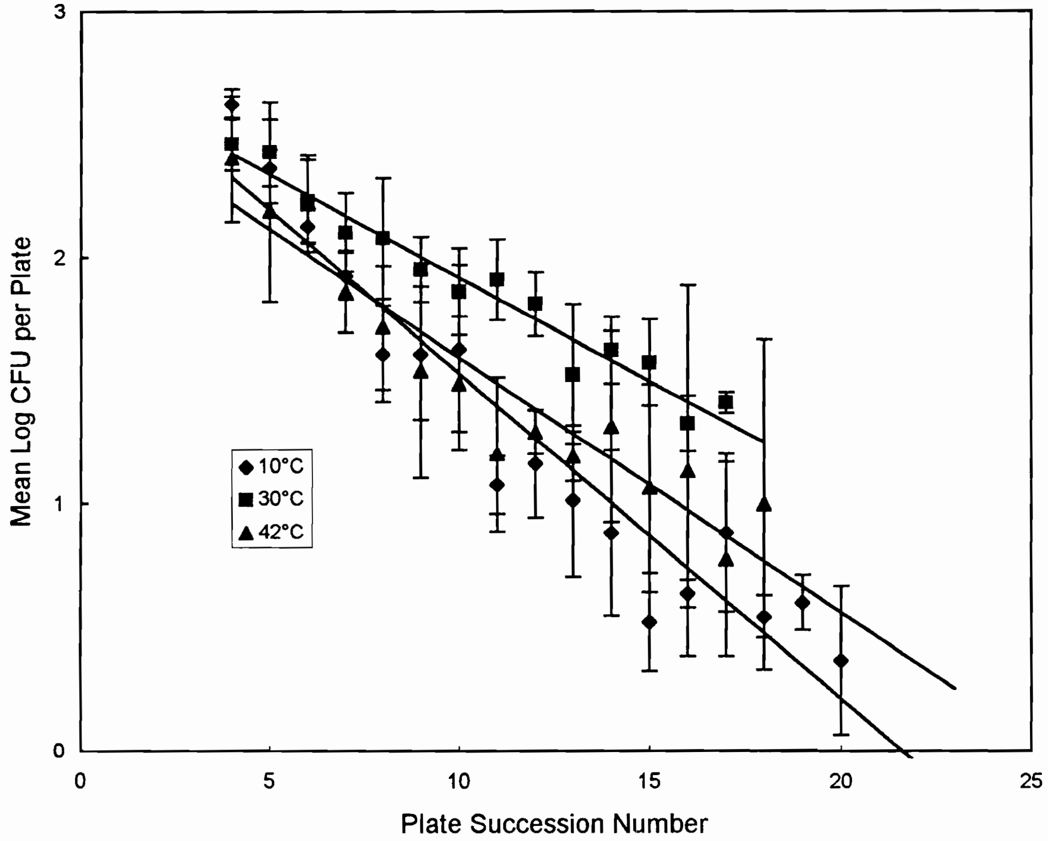

Figure 3. Detachment of Listeria monocytogenes *Scott A after growth to mid-log in TSB-YE (pH 7.0) at 10°, 30° or 42°C and exposure to Buna-N rubber for 60 min in PB (pH 7.0).*

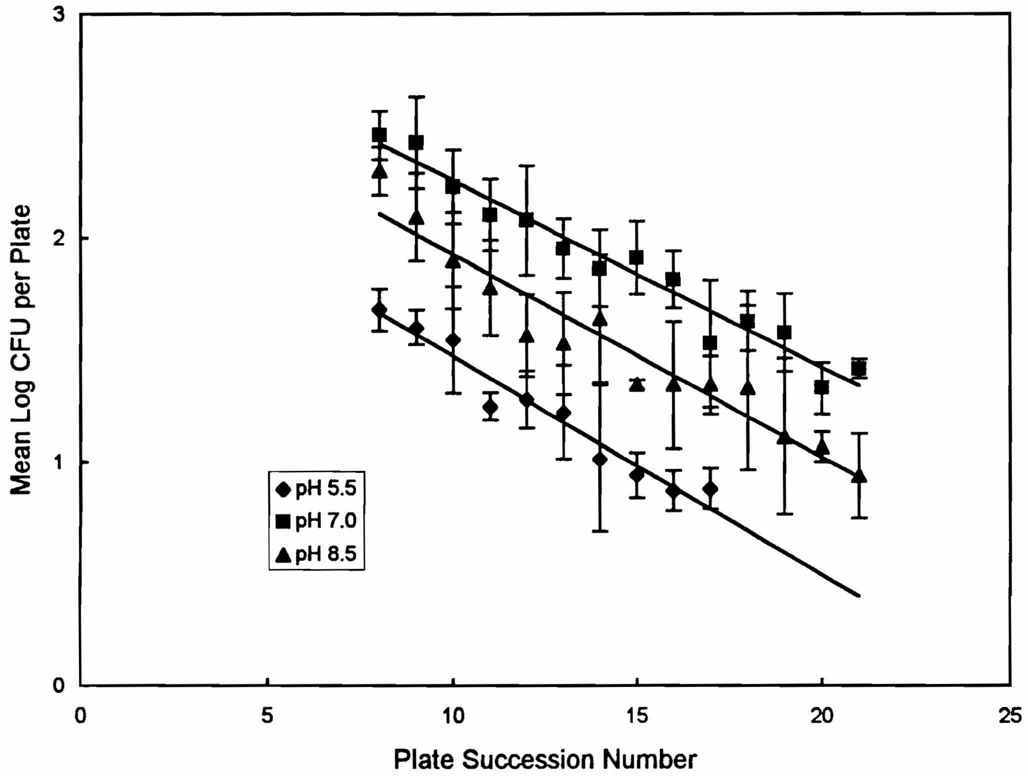

Figure 4. Detachment of Listeria monocytogenes *Scott A after growth to mid-log in TSB-YE adjusted to either pH 5.5, 7.0, or 8.5 and exposure to Buna-N rubber for 60 min in PB (pH 7.0).*

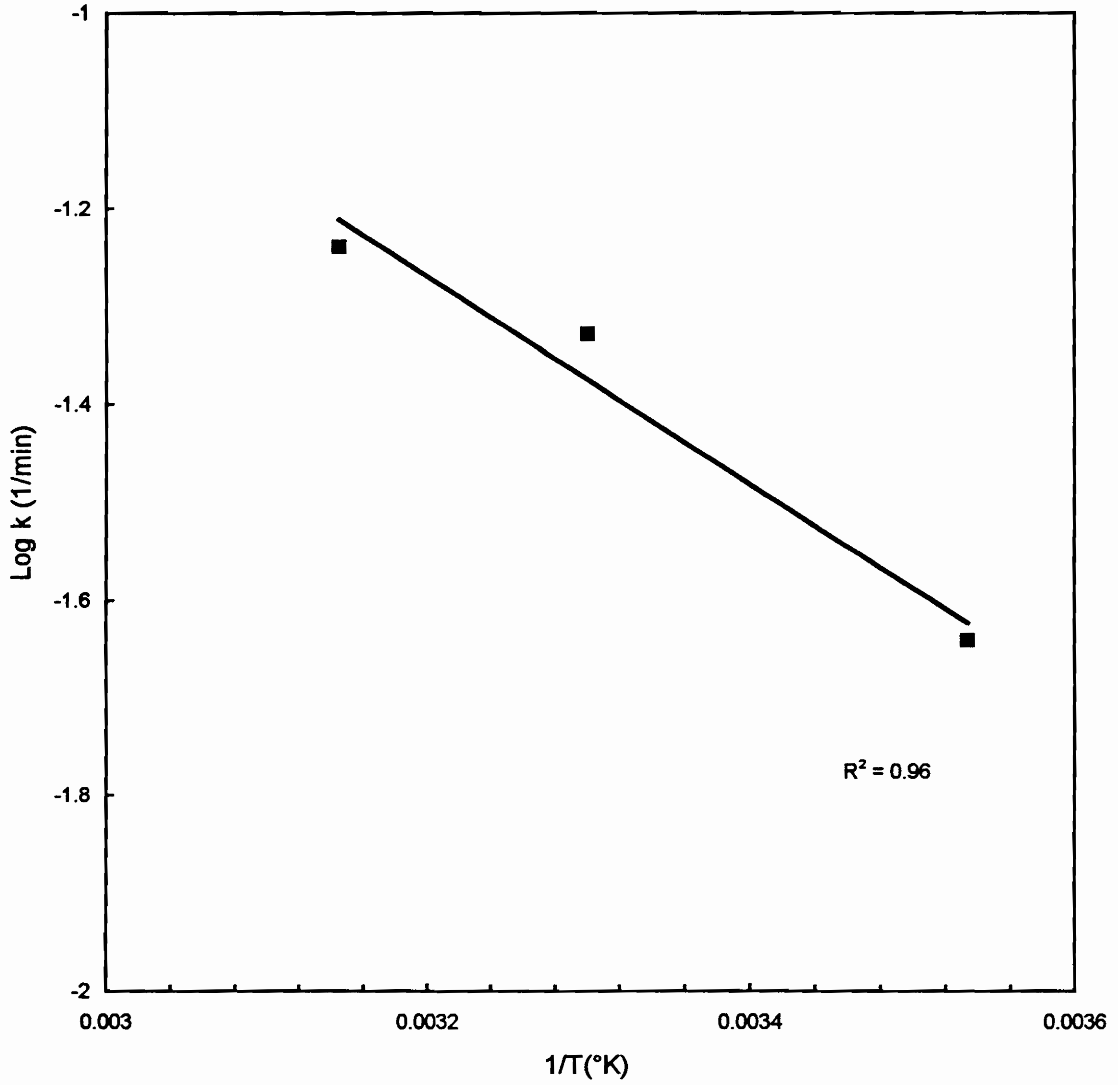

Figure 5. Arrhenius plot for the attachment of Listeria monocytogenes *Scott A to Buna-N rubber at 10°, 30°, and 45°C.*

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