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Attachment of *Listeria monocytogenes* to Stainless Steel, Glass, Polypropylene, and Rubber Surfaces After Short Contact Times

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ABSTRACT

This study was carried out to investigate the attachment capabilities of *Listeria monocytogenes* strain Scott A to stainless steel, glass, polypropylene, and rubber surfaces after short contact times at ambient (20°C) and cold storage temperatures (4°C) using scanning electron microscopy technique. Surface energy value of each surface was estimated by contact angle measurements. All surfaces displayed many possible harborages for *L. monocytogenes* attachment. Our results indicated that *L. monocytogenes* cells could attach to all surface types at both temperatures after contact times as short as 20 min or 1 h. Extracellular materials could be observed on the surfaces especially polypropylene and glass incubated at 4 and 20°C for 1 h respectively.

Listeria monocytogenes is a potential human pathogen widely distributed in the environment (*7*). It represents a source of concern to the dairy industry because outbreaks of foodborne illness involving dairy products (*4,5*) such as pasteurized milk (*10*), soft Mexican-style cheese (*18*), and ice cream (*3*) have been reported. The ability of *L. monocytogenes* to multiply at refrigeration temperatures also represents a source of concern to public health. Cox et al. (*7*) showed that listeriae could be found in all types of food production environments. They were commonly found in drains from which they could be spread over floors and eventually to surfaces which may have direct contact with the food.

In natural ecosystems and under laboratory conditions, the ability of bacteria to attach to substrates is a commonly observed phenomenon (*6,13,15,28,32*). Microorganisms attached to surfaces are important as a source of potential contamination for any material coming in contact with the surfaces. The ability of attached bacteria to contaminate products can be a significant problem in such diverse industries as food processing plants and dairy farms. Equipment parts and food contact surfaces such as stainless steel, glass, polypropylene, and rubber are widely used in the food processing industry (*27*), but limited studies on these surfaces have been reported (*17*). Scanning electron microscopy (SEM) has frequently been used to study the attachment of bacteria to inert surfaces (*20,22,27*). The aim of the present study was to use scanning electron microscopy to investigate the attachment of *L. monocytogenes* to four milk contact surfaces (stainless steel, rubber, plastic, and glass) at ambient and cold storage temperatures under short contact time conditions. In addition, the surface energy values of the studies materials were estimated by contact angle measurements.

MATERIALS AND METHODS

Organism

Listeria monocytogenes strain Scott A (a clinical isolate) was obtained from the collection of E. P. Ewan, Laboratory Center, Tunney's Pasture, Ottawa, Ontario. Bacteria were streaked onto *Listeria* plating medium (LPM:CM 819, Oxoid, Basingstoke, England) with 10% bovine blood and incubated for 18-24 h at 37°C. A single colony was then picked and inoculated in a vial containing 5 ml of trypticase soy broth (TSB:BBL, Cockeysville, MD) with 1% yeast extract (Difco Laboratories, Detroit, MI) and incubated for 18-24 h at 37°C. A viable count was done on each suspension (TSB-YE) used in the attachment test using standard plate count agar (Difco, Detroit, MI) as described by Gilliland et al. (*16*). The total plate counts routinely measured were of 2×10^8 CFU/ml.

Test surfaces

Polished stainless steel penicylinders (type 304, SS-8, finish No. 4, 8 mm outside diam. (OD), 6 mm inside diam. (ID), and 10 mm length; Fisher Scientific Co., Pittsburgh, PA), glass (borosilicate) cylinders (8mm OD, 6 mm ID, 10 mm length; Corning Glass Works, New York), polypropylene cylinders (8 mm ID, 5 mm OD, 10 mm length; Canlab Co., Canada), and disks made of nitrile rubber (15 mm diam and 3 mm thick) were used. Rubber samples were manually cut from teat cup inflation (Alfa Laval, Peterborough, Ont., Canada) with a die. All materials were similar to those used in milking installations and food industries. Before each test, the cylinders (with the exception of rubber disks which were sterilized in distilled water due to formation of precipitate with asparagine contact) were placed in an aspa-

ragine solution (0.1%) for autoclaving at 121°C for 15 min to protect them against oxidation according to the AOAC use-dilution method (*2*).

Soiling surface procedures

Sterile cylinders of stainless steel and glass and rubber disks were totally immersed, whereas free floating polypropylene cylinders were vertically dipped in a vial containing 5 ml of bacterial suspension (TSB-YE) with contact times of 20 min and 1 h at 20°C and a contact time of 1 h at 4°C. After the incubation period, the cylinders and disks were removed and drained by holding them vertically against sterile filter paper (Whatman No. 2, 9 cm). They were manually rinsed three times for 1 min each time in 0.1 M sterile cacodylate buffer (pH 7.0) to remove unattached organisms before the cells on the surfaces were fixed for scanning electron microscopy observation.

Scanning electron microscopy

In preliminary work carried out to observe the surfaces of all four types of material, the uninoculated sterile surfaces were gold coated only. The soiled surfaces (except rubber disks which were dehydrated by drying at 37°C for 30 min) were fixed by immersion in glutaraldehyde (2.5% v/v) in 0.1 M sterile cacodylate buffer, pH 7.0, for 4 h and washed twice in 0.1 M sterile cacodylate for 20 min. Post-fixation was done in osmium tetroxide (2% w/v) in sterile cacodylate buffer for 30 min at 21°C, and dehydration through an ascending series of acetone concentration (10%, 30%, 50%, 70%, 90%, and finally three times with 100%) was carried for 15 min at each step. Dehydration was completed using CO_2 in a critical point dryer (Model E 3000 CPD, Bio-Rad, Polaron Equipment Ltd., Watford Hertfordshire, England). Specimens were mounted on stubs and covered with 15 nm of gold using a sputter coater (Emscope, Bio-Rad). A Nanolab LE 2100 (Vickers Instruments, Bausch and Lomb Ltd., Ontario, Canada) scanning electron microscope operating at 15 kV was used to examine the food contact surfaces.

Contact angle measurements

The same commercial available surfaces such as stainless steel, glass, polypropylene, and rubber, described above, were used throughout the contact angle measurements.

Following sterilization, the surfaces were aseptically removed and dehydrated in sterile petri dishes matted with two layers of Whatman No. 2 filter paper. Thereafter, they were dried in a desiccator containing drierite (Hammond Drierite Co., OH) for 24 h to reduce possible surface moisture.

The dried surfaces were positioned on a microscope stage for contact angle measurements. A micro-syringe (Chromatographic Specialities Inc., Brockville, Ont., Canada), at the extremity of which a square-cut Teflon tip was fitted, was used to place drops (1 µl) of deionized water or α-bromonaphtalene (Aldrich Chemical Co. Inc., Milwaukee, WI) on solid surfaces. The contact angle was measured within 5 s using a goniometer in conjunction with a 100 x telescope (Gaertner Scientific Corp., Chicago, IL) and estimated within $\pm < 2^{o}$. In all cases, each contact angle reported in the present study was the average from at least five measurements. At least 40 measurements were taken with both wetting agents.

For the studied surfaces, the total surface energy (γTOT) and the Lifshitz-Van der Waals (LW) and the short range (SR) hydrogen bonding components of surface energy have been derived from contact angle determinations with water and α-bromonaphthalene and with the help of an extended Young equation (*31*).

RESULTS

Scanning electron microscopy (SEM) examination of two nonporous surfaces (stainless steel and glass) and two porous surfaces (polypropylene and rubber) are shown in Fig. 1 A,B,C, and D, respectively. Evaluation of these surfaces with SEM reveals that the glass surface (Fig. 1B) is smoother than the others, whereas the stainless steel surface is often marked by grooves and crevices (Fig. 1A). Nevertheless, the glass surface may possess scratches and depressions (Fig. 1B) in which bacteria might be harbored. The polypropylene surface is smooth but some particles and small holes appear to be encrusted into the surface (Fig. 1,2-3C). The rubber has a surface in which deep holes, crevices, and particles are observed (Fig. 1-4D).

Table 1 shows the surface energy values of the four studies surfaces estimated by contact angle measurements

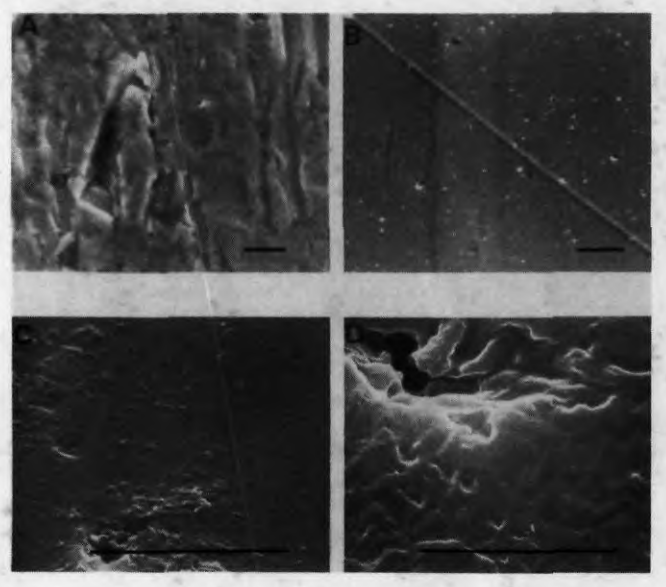

Figure 1. *Scanning electron photomicrograph of new food contact surfaces. (A) stainless steel, (B) glass, (C) polypropylene, (D) rubber. Bar: 5 µm.*

using water and α-bromonaphthalene. The difference between the flat surfaces and the penicylinders in terms of total surface energy values was relatively low (data not shown). Rubber and polypropylene were low energy surfaces as compared to stainless steel and glass. However, the Lifshitz-Van der Waals (LW) contributions of the stainless steel, glass, and polypropylene surfaces were similar, whereas the short range hydrogen bonding components (SR) of the stainless steel and glass surfaces were very high as compared to those of polypropylene and rubber.

Figures 2-4 are electron micrographs of contact time experiments at ambient (20°C) and cold storage temperatures (4°C) in which *L. monocytogenes* strain Scott A was allowed to attach to stainless steel, glass, polypropylene, and rubber surfaces. SEM examination revealed that *L. monocytogenes* could attach to both porous and nonporous

TABLE 1. *Contact angles and surface energy of the used surfaces.*

Surface	Contact angle, degree: Water	SD[1]	α-bromonaphthalene	SD	Surface energy, mJ.m^{-2}: γ^{SR}	γ^{LW}	γ^{TOT}
Stainless steel penicylinder	58.2	1.5	21.2	1.0	15.3	35.0	50.3
Glass cylinder	64.1	4.6	27.1	3.6	12.4	33.9	46.3
Polypropylene cylinder	89.9	0.8	20.6	2.9	0.9	39.9	40.9
Rubber disk	106.0	2.7	25.2	1.5	0.3	23.8	24.1

[1]SD: Standard deviation.

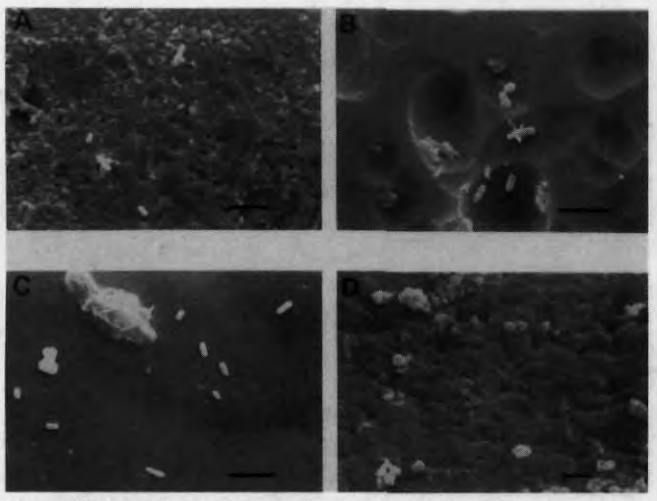

Figure 2. *Scanning electron photomicrograph of* Listeria monocytogenes *Scott A cells attached to (A) stainless, (B) glass, (C) polypropylene, and (D) rubber after 20 min of contact time at 20°C. Bar: 3 μm.*

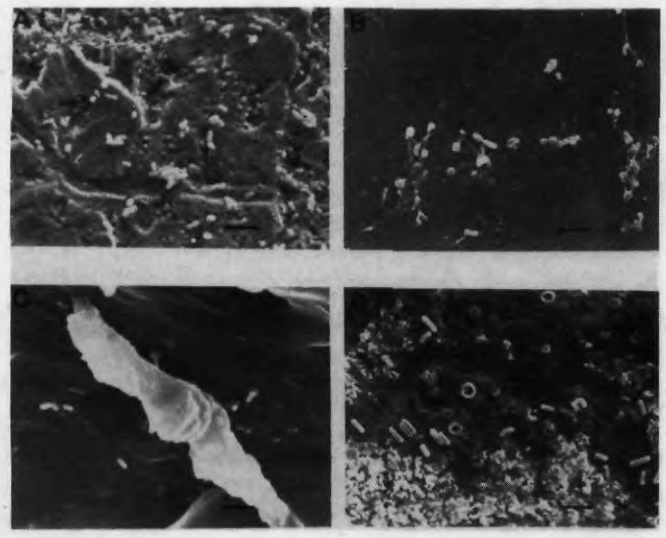

Figure 3. *Scanning electron photomicrograph of* Listeria monocytogenes *Scott A cells attached to (A) stainless steel, (B) glass, (C) polypropylene, and (D) rubber after 1 h of contact time at 20°C. Bar: 3 μm.*

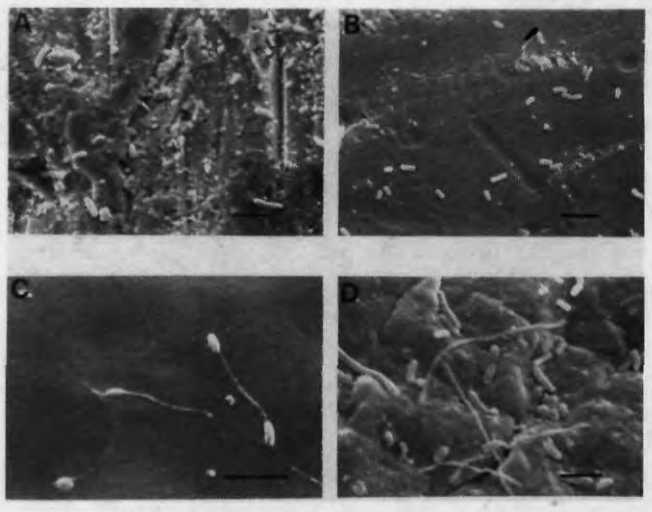

Figure 4. *Scanning electron photomicrograph of* Listeria monocytogenes *Scott A cells attached to (A) stainless steel, (B) glass, (C) polypropylene, and (D) rubber after 1 h of contact time at 4°C. Bar: 3 μm.*

surfaces after short contact times (Fig. 2-4). The grooves and crevices of stainless steel and rubber surfaces appeared large enough to fit *L. monocytogenes* cells (Fig. 2-4A and D).

At ambient temperature, attachment of few *L. monocytogenes* cells to the surfaces was observed after 20 min (Fig. 2). Under this incubation condition, single cells were observed to be attached to all surfaces without the aid of visible fibrils or extracellular materials. After a contact time of 1 h at 20°C (Fig. 3), extracellular materials appeared to surround *L. monocytogenes* cells attached to the glass surface (Fig. 3B).

At 4°C for 1 h, it is interesting to note that the presence of extracellular materials surrounding *L. monocytogenes* cells attached to glass and polypropylene surfaces was also observed (Fig. 4B and C).

DISCUSSION

Deposition of bacterial cells onto solid surfaces may result in proliferation and subsequent colonization of these surfaces under favorable conditions. In the present study, SEM examination has demonstrated that *L. monocytogenes* strain Scott A could attach to surfaces after contact times as short as 20 min or 1 h at ambient and cold storage temperatures, respectively. Surface irregularities were observed (Fig. 1) and physical entrapment of bacteria in the harborage of the surfaces would seem to enhance attachment. However, no correlation was found between the surface irregularities of material and the ability of *L. monocytogenes* to attach to that particular surface.

The attachment of *L. monocytogenes* cells to stainless steel, glass, polypropylene, and rubber surfaces did not show an increasing trend with longer contact times (20 min versus 1 h), but the presence of extracellular materials surrounding attached cells to surfaces such as glass,

polypropylene, and rubber was observed after a contact time of 1 h at both temperatures. Our results appear to be in agreement with the findings of previous studies on bacterial attachment. Gelinas and Goulet (*15*) found that *Pseudomonas aeruginosa* could attach to stainless steel, aluminum, and polypropylene after 15 min. In addition, these authors noted that the presence of extracellular materials was observed between attached cells to polypropylene after contact times of 1 and 3 h. Costerton et al. (*6*) stated that many bacterial cells, in their competitive environment, secrete a long polysaccharide fiber which serves in attaching the organisms to a surface. Some strains of *L. monocytogenes* were reported to possess a polysaccharide capsule that may be related to virulence (*26*). It was demonstrated that a primary acidic polysaccharide may be responsible for initial bacterial adhesion and that a secondary fibrous acidic polysaccharide may probably appear from it after initial attachment (*13*).

It is also possible that the fibrillar appearance of extracellular polymer produced by surface-associated bacteria may be attributed to condensation and chemical denaturation during specimen preparation before examination by SEM (*21*). More specially, Fraser and Gilmour (*14*) have attributed fibril formation to the ethanol dehydration stage of the SEM chemical preparation procedure. Thus, the extracellular material observed at 4 and 20°C may be the result of chemical dehydration, but it is also possible that the polymeric materials played some role in the attachment of *L. monocytogenes* to the surfaces. In addition, Fletcher (*11*) indicated that fibrils may be beneficial to the cells by increasing contact with the surface by overcoming any electrostatic repulsion force with their kinetic energy.

Herald and Zottola (*17*) and Mustapha and Liewen (*24*) found that *L. monocytogenes* strains could produce vast amounts of polymeric materials when adhering to stainless steel surfaces during longer incubation times than those studied in this work. Moreover, Herald and Zottola (*17*) noted that extracellular materials were observed only at 21°C and not at 35 or 10°C. Our results showing that *L. monocytogenes* could produce polymeric materials at 4°C is in agreement with Stone and Zottola (*29*) who reported that the formation of attachment fibrils by *Pseudomonas fragi* was not affected at 4°C. Costerton et al. (*6*) noted that the polysaccharide fiber may act as protector of the cells from outside stress such as cold temperatures.

Extracellular materials which may be neutral or negatively charged polysaccharides may adsorb to the surface to form a polymer bridge or increase the tendency of some bacteria to attach by reducing electrostatic repulsions between an organism and a surface (*19*). In several studies the adhesive property of various inert surfaces for microorganisms and the nature of the contact to these surfaces have been investigated. Many authors have reported that the ability of a surface to absorb macromolecules is related to surface tension, surface charge, and various other physical factors such as pH (*8,9,12,23,25*). These factors can affect the rate of bacterial attachment.

Surface charge depends upon the aqueous environment as well as the material of the solid. However, in practice, the electrostatic force tends to become negligible for the adhesion of particles to surfaces in the presence of a liquid medium because of the enhanced dielectric constant of the liquid medium and the adsorption phenomena involved which tend to shield the charge of the adherents (*30*). Hence, factors other than the surface charge of the studied materials might be responsible for attachment of *L. monocytogenes* cells.

The results from this work have shown that *L. monocytogenes* strain Scott A can attach to four surfaces after short contact times at ambient and cold storage temperatures. The attachment mechanism of *L. monocytogenes* is not yet known although attachment capabilities may be associated with the presence of extracellular materials. Nevertheless, attachment of *L. monocytogenes* may also be affected by the intrinsic properties of the surface.

Interfacial forces may be important factors involved in attachment of *L. monocytogenes* cells. Interfacial properties of surfaces estimated by contact angle measurements indicate that rubber disks were the lowest energy surfaces, whereas stainless steel penicylinders were the highest (Table 1). Absolom et al. (*1*) noted that bacterial adhesion is more extensive to hydrophobic surfaces of relatively low surface energy than to hydrophilic when the surface tension of the suspending medium is larger than that of bacteria. These authors have shown that the surface energy of *L. monocytogenes* was slightly lower than that of water (66.3 $mJ.m^{-2}$).

The attachment capabilities of *L. monocytogenes* observed in this study are leading us to the investigation of the efficacy of sanitizers used in the food industry to achieve the destruction of any attached *Listeria* cells.

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