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AN ELECTRON MICROSCOPIC STUDY OF THE ADHERENCE OF LACTOBACILLUS ACIDOPHILUS TO HUMAN INTESTINAL CELLS IN VITRO

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Abstract

In this study, transmission electron microscopy was used to visualize the adherence of Lactobacillus acidophilus to human intestinal tissue cells (HITC) in vitro. There appeared to be a layer of electron dense material on the bacterial cell and on the intestinal cell which may mediate adherence. When L. acidophilus attached to intestinal tissue cells after a short contact period, it did not appear to disrupt the integrity of the intestinal cell. Treatment of the bacterium with sodium periodate and Concanavalin A reduced the adherence to HITC, suggesting that a carbohydrate was involved. Electron micrographs of periodate-treated cells revealed that the layer appeared to be partially removed after 1 hour and was almost completely absent after 3 hours of treatment.

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Introduction

Lactobacillus species are used in the production of numerous foods including yogurt, acidophilus milk, kefir, Italian cheese, sourdough bread, distillery mash, pickles, olives, and some cured meats. Milk is the substrate for several lactic acid fermentations involving the lactobacilli. One such product is acidophilus milk. It, however, has not received a great deal of consumer acceptance as it has a very sour flavor and high lactic acid content. Metchnikoff (1908) speculated that consuming "sour milk" could terminate the unhealthy fermentation going on in the gut and lead to a longer life. Several investigators have suggested that L. acidophilus may have other beneficial effects for humans. These would include cholesterol assimilation, cancer suppression, and antibiotic production (Sandine, 1979; Gilliland et al., 1985; Goldin and Gorbach, 1984).

Methods have been proposed to make products containing high numbers of L. acidophilus cells without the undesirable taste. One of these products is "Sweet Acidophilus Milk." One method of producing this product is by adding high numbers of freeze-dried viable cells to cold (5°C) pasteurized milk (Speck, 1978). The cells remain viable in the cold milk but do not grow until the milk is consumed. A novel product consisting of acidophilus milk blended with banana, tomato juice, and sugar and then spray-dried has also been suggested (Prajapati et al., 1986).

Brownlee and Moss (1961) first demonstrated that lactobacilli adhere to stomach epithelial cells in the rat stomach. Schaedler et al. (1965) noticed that lactobacilli isolated from normal mice immediately established themselves throughout the gastrointestinal tract of germ-free mice. In another example, Fuller and Turvey (1971) found that the main sites of attachment of lactobacilli in the chicken gastrointestinal tract were the crop, ileum, and caecum. The adherence of the lactobacilli was unaffected by diet, and they suggested that there was a great degree of specificity between the microorganisms and host because only avian lactobacilli would adhere to chicken crop epithelial cells.

Other investigators have suggested species-specificity in the adherence of the lactobacilli. Barrow et al. (1980) found that only strains of Lactobacillus fermentum and Streptococcus salivar isolated from pigs and wild boars would adhere to pig epithelial cells. In another example, lactobacilli isolated from pigs and fowl did not adhere to keratinized squamous epithelium cells of the mouse stomach, while a rat

isolate did adhere (Wesney and Tannock, 1979). Conway et al. (1987), in a comprehensive study, determined the survival of four strains of lactic acid bacteria in human gastric juice, in vivo and in vitro, and in pH 1 to 5 buffered saline. In addition, they studied the adherence of two strains of Lactobacillus acidophilus, L. bulgaricus, and Streptococcus thermophilus to freshly collected human and pig small intestinal cells and to pig large intestinal cells. They demonstrated that the ability of these four microbes to survive in gastric juices and adhere varied significantly. In their study, survival and adherence was enhanced by milk, and all strains tested adhered to some extent to both human and pig intestinal cells. They suggest that the adhesion mechanism is probably nonspecific, which is in conflict with other reported specific adhesion of lactobacillus (Barrow et al., 1980; Kotarski and Savage, 1979; Mayra-Makinen et al., 1983). Therefore, it is important to study the adherence of organisms to the species of interest.

The objectives of this study were to use electron microscopy to 1) examine the adherence of L. acidophilus to human intestinal cells, and 2) to investigate mechanisms by which L. acidophilus adheres to human intestinal cells.

Materials and Methods

Bacterial strains and media

L. acidophilus BG2F04 (obtained from T. Klaenhammer, North Carolina State University) was grown by daily transfer in MRS broth (Difco) incubated at 37°C. The identity of the L. acidophilus was confirmed by determining carbohydrate fermentation patterns using the Rapid CH Strip (dms/laboratories; ordered from API, Plainview, NY).

Tissue culture

Human intestinal tissue cells (HITC) FHs0074 (ATCC CCL 241) were cultured in a monolayer on Falcon plastic tissue flasks (25 cm^2 surface area). For adherence work, the cells were used at passage levels of 21 to 25. Growth medium consisted of Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 0.2 units/ml insulin (Sigma), 1 mM oxalacetic acid (Sigma), 0.5 mM sodium pyruvate (Sigma), 0.1 mM non-essential amino acids (GIBCO), and 30 ng/ml epidermal growth factor (GIBCO). The cells were incubated at 37°C in a 5% CO_2 atmosphere and were allowed to grow confluent (approximately 1 week of growth) before use. Spent medium was removed and replaced with new medium every 2-3 days.

To determine relative adherence, the cells were cultured in plastic Leighton tubes (Costar Labs., Cambridge, MA). For electron microscopy, the tissue cells were grown in the Falcon plastic culture flasks.

Adherence study

The method of Kleeman and Klaenhammer (1982) was followed. Bacteria were incubated with the tissue cells for 20 min. Earle's balanced salt solution (EBSS) without calcium and magnesium (GIBCO) was used as the rinse and suspending medium. Preparations were examined using a Nikon Labphot microscope at a magnification of 400X.

Effect of chemical treatments on adherence

The effect of several treatments on the adherence of L. acidophilus BG2F04 was investigated by suspending the organism in solutions prior to adherence testing. The treatments included sodium periodate (10 mg/ml) (Sigma), Concanavalin A (0.2 mg/ml) (Sigma), EDTA (4.5%) and ruthenium red (RR) (0.05%) (EMS, Port Washington, PA). All treatments except EDTA were carried out at 37°C for 1 h. The EDTA treatment was done just prior to adding the bacterial suspension to the human intestinal tissue cells. Relative adherence was measured by counting the number of bacteria per tissue cell and dividing that number by the number of bacteria adhered to in an untreated control.

Transmission electron microscopy

To examine the cellular morphology of cells growing in broth, the bacterial cells were grown as previously described in 30 ml MRS broth, collected by centrifugation (3000 x g for 10 min), and rinsed once by resuspending the pellet in 0.1 M sodium cacodylate buffer (pH 7.2). The cells were collected by centrifugation (3000 x g for 10 min) and prepared for electron microscopy as described below. The effect of sodium periodate was studied by suspending the cells in a sodium periodate solution and incubating 1 and 3 h at 37°C.

Fixation

Primary fixation was done with 2.5% glutaraldehyde (EMS) in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature. Secondary fixation was done with 2% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h at room temperature.

Ruthenium red

For staining, 0.1% RR (EMS) was mixed with double strength primary fixative in a ratio of 1:1. The final concentration of RR was 0.05%.

Colloidal iron

After primary fixation the samples were treated with a mixture of distilled water, glacial acetic acid, and stock iron solution (Mowry, 1963) in the ratio 9:6:5 for 1 h (Brooker and Fuller, 1975). The pH of the solution was 1.8.

Dehydration, embedding, and post stains

The cells were collected, dehydrated in graded acetone series (5 min each in 25%, 50%, 75%, 95%, and 3 times in 100%) and infiltrated with 4:1 acetone to Spurr's resin (cured for 20 h at 60°C), sectioned with glass knives, and collected on Formvar-coated, copper grids. Post staining was done with lead citrate (3 min) and uranyl acetate (20 min). All samples were viewed on a Philips 300 transmission electron microscope.

To examine bacterial adherence to the tissue cells, the bacteria were prepared as described for adherence testing. The suspension was allowed to contact the tissue cells for 20 min, at which time the monolayer was rinsed twice with EBSS and once with sodium cacodylate buffer. The monolayer was fixed, dehydrated in a graded ethanol series, infiltrated, and embedded in situ. After the resin was cured, blocks were cut with an electric jig saw and sectioned and stained as previously described.

Scanning electron microscopy

Sample tissue cells with adhered bacteria were prepared by placing a sterile, round glass coverslip in the tissue culture flask. The cells were allowed to grow confluent over the coverslip, and then the method of Kleeman and Klaenhammer (1982) was followed to allow the bacteria to adhere to the tissue cells. The monolayer was rinsed twice with EBSS and once with 0.1 M sodium cacodylate buffer. Samples were fixed in 2.5% glutaraldehyde containing 0.05% RR for 1 h at room temperature.

The monolayer was rinsed again and then fixed in 2% OsO_4, the coverslip was removed with a forceps and placed in a holder. Primary dehydration was done at room temperature using a graded acetone series (5 min each in 25%, 50%, 75%, 95%, and 3 times in 100%). Secondary dehydration was done by critical point drying using CO_2 as the transition medium. The coverslips were mounted on aluminum stubs and coated with gold-palladium in a Kinney vacuum evaporator (model KSE2-AM). The samples were viewed in a Philips 500X scanning electron microscope at 12 kV.

Results and Discussion

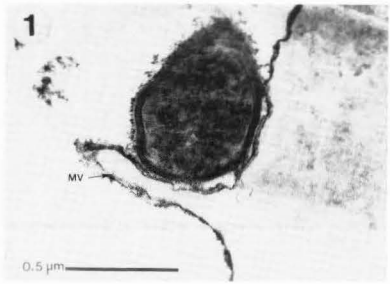
Adherence testing

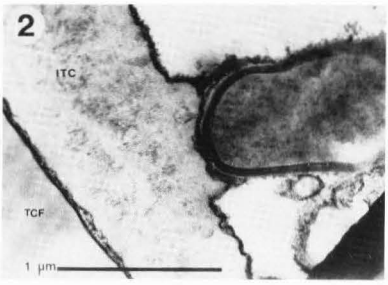
When selecting lactobacilli to be used in food for their possible therapeutic value, factors concerning the capabilities to perform desired roles should be considered (Speck, 1980). One of those factors may be the ability of the organism to associate with and colonize in the intestinal tract. Methods using tissue culture have been suggested as a means for examining the adherence of bacteria to eukaryotic cells (Hartley et al., 1978). However, it should be noted that the environment of the intestine differs greatly from tissue culture medium used in vitro.

L. acidophilus BG2F04 has been shown to strongly adhere to human intestinal and tissue cells (Kleeman and Klaenhammer, 1982). Previous research in our laboratory revealed that strain BG2F04, when stained with RR, showed an electron dense layer exterior to the cell wall (Hood and Zottola, 1987). RR is commonly used to stain carbohydrate material but may also react with lipids (Luft, 1971). When L. acidophilus BG2F04 was allowed to adhere to HITC, electron microscopy showed a dark layer of material which could be seen on the bacterial cell as it attached to the intestinal cell (Fig. 1). There also appeared to be an electron dense layer on the HITC. It is common to find an acid mucopolysaccharide on the surface of mammalian cells (Behnke, 1968). It may be the association of the two surface layers that mediates adherence.

While the microorganism appeared to be very closely associated with the HITC, it did not appear to disrupt the integrity of the HITC. In Fig. 2, it appeared that the HITC had conformed to the shape of the bacterial cell. This may been an attachment site on the HITC, or it may just have been an artifact of the preparation procedure. Ward and Watt (1972) described similar "cushion-like" structures in electron micrographs of Neisseria gonorrhoeae adhering to urethral epithelial cells.

There is substantial evidence to indicate that the adherence of lactobacilli to mucosal surfaces is mediated by a polysaccharide on the surface of the cell. In a study using electron microscopy to observe bacterial adherence, Brooker and Fuller (1975) employed ruthenium red, colloidal iron, and alcian blue stains to determine the composition of the material associated with the adherence of lactobacilli to chicken crop epithelium. The material stained dark or were electron dense with these dyes. Curren et al. (1965) and Luft (1971) have shown that these stains are specific for polysaccharides and consequently can be used for identifying complex carbohydrates as cell components utilizing electron microscopy. When colloidal iron was used to stain the cells in this study, an electron dense layer was seen exterior to the cell wall (Fig. 3). The colloidal iron method is another technique which has been used to





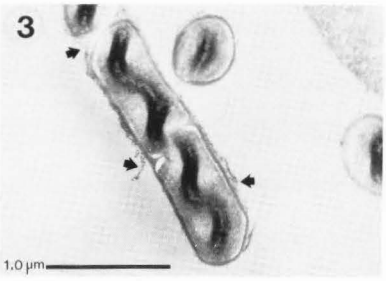


Figure 1. TEM of L. acidophilus BG2F04 adhered to HITC. Note the layer of material on the intestinal cell. MV: microvilli

Figure 2. TEM of L. acidophilus BG2F04 on HITC. ITC: intestinal tissue cell; TCF: tissue culture flask

Figure 3. TEM of L. acidophilus BG2F04 fixed using the colloidal iron method. Arrows indicate the electron dense layer exterior to the cell wall.

identify acidic polysaccharides. This stain is specific for carboxyl groups that would be found in such a carbohydrate. It will also stain other molecules which contain carboxyl groups. The results presented here suggest the involvement of a polysaccharide layer and agrees with the results shown by Brooker and Fuller (1975).

Conway et al. (1987) in their study of the adherence of four different strains of lactic acid bacteria to both human and pig intestinal cells in vitro utilized radioactively labelled bacterial suspensions to determine adherence. They estimated the number of bacteria bound to the intestinal cells from the bacterial specific activity. No attempt was made by these authors to visualize adherence by using electron microscopic techniques. The amount of manipulation done with the bacterial suspensions and the intestinal cells from both pigs and humans makes one wonder if what they observed was indeed adherence and not an artifact created by the techniques used. Their data are contrary to other investigators (Barrow et al., 1980; Kotarski and Savage, 1979; Maya-Makinen et al., 1983) who clearly demonstrated the specificity of adherence of lactobacilli to mammals other than humans. Although only one strain of L. acidophilus was used in the results presented here, it is also in disagreement with the findings of Conway et al. (1987), as adherence appeared to be specific in this study.

Scanning electron microscopy

Many gram-negative organisms, such as Salmonella, produce extracellular fibrils to aid in their attachment to solid surfaces (Schwach and Zottola, 1982). This phenomenon was also observed when organisms thought to be lactobacilli adhered to chicken crop cells (Brooker and Fuller, 1975). When L. acidophilus BG2F04 was viewed on HITC, attachment fibrils were not detected (Fig. 4). The surface of the HITC is not smooth, so some bacterial cells may be trapped by microvilli and foldings on the surface of the HITC. From these results, it appeared unlikely that fibrils were involved in the adherence of L. acidophilus to the human gastrointestinal tract.

Effect of chemical treatments on adherence

Electron microscopy provided evidence that an acidic polysaccharide was present on adhering strains of L. acidophilus. The adhering strain BGF204 was treated with compounds which have the ability to alter, in some way, the structure of carbohydrates and possibly polysaccharides. The results are summarized in Table 1. Sodium periodate greatly reduced the ability of L. acidophilus BG2F04 to adhere to HITC. Fuller (1975) reported that periodate had a similar effect on the adherence of lactobacilli to chicken crop cells. Periodate is a strong oxidizing agent and has the capacity to cleave C-C bond of 1,2 diols that occur in sugars and which may be constituents of a polysaccharide (Hay et al., 1965). It has also been used in light microscopy in combination with Schiff's reagent to stain polysaccharides. Lipoteichoic acids (LTAs) also have been indicated as a component of the cell that may be involved in adherence (Sherman and Savage, 1986). Since LTA's contain glucose side groups, it is possible that periodate is disrupting the LTA's. In either case, this evidence points to a carbohydrate component on the cell surface that is responsible for the ability of the organism to adhere.

Electron micrographs of L. acidophilus treated with sodium periodate revealed that, after 1 h of exposure (Fig. 5A), the exterior layer was still present, although it was not as dense as the layer seen in Fig. 1. When treated for 3 h, it appeared that the layer was almost completely absent (Fig. 5B). It would seem that after 1 h, the component of the cell responsible for adherence was changed sufficiently to inhibit adherence, although the putative polysaccharide layer is still partially present. This suggested that the chemical structure of this layer was important for adherence.

Con A also appeared to reduce the ability of L. acidophilus to adhere to HITC, although the reduction was not as great as that by periodate (Table 1). Steinberg and Gepner (1973) suggested the use of Con A as a means to identify sites of adhesion which involve carbohydrates. Con A specifically binds to polysaccharides containing alpha-D-glucopyranosyl, alpha-D-mannospyranosal, or certain 5-carbon rings and thus may be blocking sites of adherence on the bacterial cell.

Ruthenium red did not appear to inhibit the adherence of L. acidophilus to HITC. As previously stated, RR is used to stain acidic polysaccharides, so it might be expected to reduce adherence. The fact that adherence was not reduced may be an indication that the carboxyl groups involved in ionic binding with RR were not important in the adherence of L. acidophilus to HITC.

Table 1. Effect of various chemical treatments on the relative adherence of L. acidophilus to human intestinal cells in tissue culture.

Treatment	Relative Adherence*
Control	1.0
Sodium Periodate	0.03
Ruthenium Red	0.99
Con A	0.74

*Relative adherence = no. bacteria per HITC / no. bacteria control HITC

Summary

There appeared to be a layer of electron dense material on the intestinal cell, and the bacterium may associate with this layer when adhering. When L. acidophilus attached to intestinal tissue cells after a short contact period, it did not appear to disrupt the integrity of the intestinal cell.

Treatment of the organism with sodium periodate and Con A reduced the adherence to HITC, also indicating that a carbohydrate was involved. Although RR did not inhibit adherence, it is possible that RR did not bind to the functional groups involved in adherence. Electron micrographs of periodate-treated cells revealed that the layer appeared to be partially removed after 1 h and was almost completely absent after 3 h of treatment.

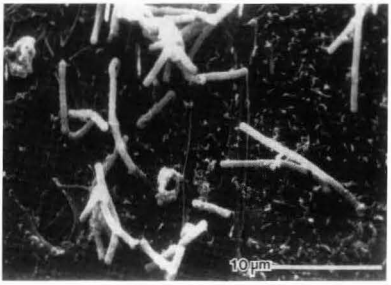


Figure 4. SEM of L. acidophilus BG2F04 on HITC. Attachment fibrils are not produced by the bacterial cell. Note the rough texture of the intestinal cell.

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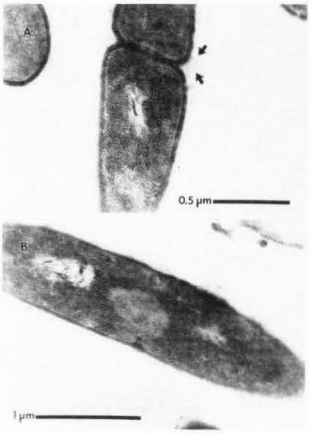

Figure 5. TEM of L. acidophilus BG2F04 treated with sodium periodate for A) 1 h, and B) 2 h.

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Discussion with Reviewers

T.A. McMeekin: What is the nature of the fibrils observed in earlier work with gram-negative bacteria? Are these simple extensions of the outer membrane?

Authors: The nature of the fibrils of gram-negative bacteria is addressed in another manuscript soon to be published in the Journal of Food Science (Herald and Zottola).

T.A. McMeekin: Is there species specificity involved in the adherence of Lactobacillus acidophilus to HITC? Do the results of your previous publication (Hood and Zottola, 1987, text reference), in which L. acidophilus adhered to glass, suggest that the carbohydrate layer mediates non-specific adhesion?

Authors: We feel that species specificity is involved in the adherence of L. acidophilus to HITC. In our previous study several different strains of L. acidophilus were examined for adherence properties. Several were able to adhere to glass that lacked the carbohydrate layer as defined in the paper here. This suggests that there is another non-specific mechanism for adherence to inert surfaces. Only one strain was used in the results given in this paper and in this instance species specificity seems to be involved.

T.A. McMeekin: Figure 2, and possibly Figure 1, indicate polar adhesion of L. acidophilus to HITC. Was this orientation seen consistently? Is there a specific adhesion site at the pole, or is it more hydrophobic than rest of the cell? Figure 2 is reminiscent of micrographs by Marshall and Cruickshank (1973), Arch. Mikrobiol. 91:29, showing polar orientation of bacterial cells at surfaces.

Authors: Polar adhesion was observed frequently but, at this time, it is not known if specific adhesion sites exist at the poles.

P. Allan-Wojtas, M. Kalab: The list of references should be expanded. JW Costerton is well known in the field of bacterial attachment and his work, in particular following two of his papers should be included and discussed. (1) Costeron JW, Geesey GG, Cheng KJ. (1978). How bacteria stick. Scientific American 238, 86-95; and (2) Costeron JW, Irvin, RT. (1981). The bacterial glycocolyx in nature and disease. Ann. Rev. Microb. 35, 299-324. The former paper is important because it discusses the fact that "in nature (but not in laboratory cultures), bacteria are covered by a "glycocalyx" of fibers that adhere to the surfaces and to other cells". This may explain why the authors failed to see fibrils on their cultured bacteria. Many books published on the subject of the manuscript have not been mentioned e.g., Boedeker EC. (1984). Attachment of organisms to gut mucosa, 1 and 2. CRC Press, Inc.

The effect of various types of preparation on the appearance of bacteria have been studied by others, e.g., Fraser TW, Gilmour A. (1986). Scanning electron microscopy preparation methods: their influence on the morphology and fibril formation in Pseudomonas fragi (ATCC 4973). J. Applied Bacteriol. 60, 527-533.

Authors: We are well aware of these references. The manuscripts from Costerons' group deal with primarily gram-negative marine organisms and gram-negative pathogenic bacteria. We did not feel these were pertinent to the study reported here and thus did not include them. Boedeker's book is a compilation of review papers from many authors, which did not appear to be applicable to the topic. Several of the authors in the book are referenced in other ways in this paper. The paper by Fraser and Gilmore is concerned with a gram-negative organism and its apparent attachment on inert material and does not appear to be relevant to this study.

P. Allan-Wojtas, M. Kalab: Wasn't there a chance that the washing of the bacteria in solutions used might have killed them and, consequently, they may not have adhered because they were dead rather than because their surface has been changed?

Authors: It is quite likely that the bacteria were killed in the washing solutions; however, in another study (Hood and Zottola, J. Food Science, in press) we report that dead L. acidophilus were able to adhere.

P. Allan-Wojtas, M. Kalab: Whose TEM techniques (fixation, dehydration, etc., ruthenium red and colloidal iron staining techniques, etc.) were followed? Dehydration is done in both acetone and ethanol, why the difference?

Authors: References are given in the methods section where appropriate. Where none are given the procedure used was developed by the authors. Acetone is used for dehydration of the bacterial cells alone. Ethanol was used when dehydrating the HITC because acetone solubilized the plastic tissue culture flasks and HITC support was lost.

P. Allan-Wojtas, M. Kalab: Why were blocks cut with an electric jig saw rather than trimmed with a

glass knife or steel blade?
Authors: The embedding resin was poured into the tissue culture for trimming with a steel blade, the flasks had to be cut apart into small blocks. This was accomplished very efficiently with an electric jig saw.

P. Conway: From Figure 4, the authors detected no fibrils. Their assumption is therefore that fibrils may not be involved in the human GI tract. Their sample preparation may not have preserved fine fibrils and the direct extrapolation from their cell line to the human gut is a little large.
Authors: Other work carried out in our laboratory has dealt with many different gram-negative and gram-positive bacteria. The preparation methods used with these organisms were similar. Gram-negative bacteria usually show fibrils, whereas gram-positive organisms do not. We stand by our assumption that no fibrils are produced by L. acidophilus.

P. Conway: The authors must also test protease treatments. While they have shown that periodate and concanavalin A inhibit, how can they be sure the adhesin is not sterically masked by the large concanavalin A molecule or that the oxidation of carbohydrates by the periodate does not destroy a nearby component which may be the adhesin? The inhibition for the concanavalin A is quite poor and as no statistics are presented, the question must be raised as to how significant the value of Con A is. If it is not significant, then the inhibition is only by the periodate. In the absence of the use also of sodium iodate as a control, the action of the periodate is questionable. Sodium periodate can contain sodium iodate which can be inhibitory without oxidizing carbohydrate groups.

Authors: The comments related to the use of chemical treatments are appreciated. They simply have not yet been done with this organism. In another study in our laboratory using the gram-negative organism, Pseudomonas fragi, this has been done and has been reported (Herald and Zottola, The effect of various agents upon the attachment of Pseudomonas fragi to stainless steel. J. Food Science, in press). In the above noted study, we did as was suggested by Dr. Conway and concluded that attachment by P. fragi is mediated by a complex polysaccharide. Eventually a similar study will be done with L. acidophilus.

P. Conway: Figure 5 shows that the electron dense material is removed by periodate, but without analysis of this released material, the authors cannot be sure they have not also released other material e.g. the adhesin. This problem was discussed by Fuller (1975) who showed that the proteolytic enzyme, pepsin, released material from the bacterial cell surface that had an affinity for concanavalin A. Material released by periodate may contain protein.
Authors: Our response to this comment is the same as given above. What is suggested has been done with P. fragi and is reported in the above noted paper. The results given in the paper by Herald and Zottola imply that a lipoprotein-polysaccharide complex is involved in the attachment of P. fragi to inert surfaces. A similar study will be done with L. acidophilus and adherence to human intestinal cells in tissue culture to see if a similar compound is involved.