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Location of Bacteria in the Mid-Colon of the Rat

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The distribution of microorganisms in the mid-colon of the rat was studied by light and scanning electron microscopy. An antiserum against rat colon mucus was used to stabilize the mucus in situ. In samples not incubated with antiserum, the mucus disintegrated and contracted into patchy strands only partly covering the luminal surface of the colon. Bacteria were seen within fecal pellets, tangled among the strands of mucus, and scattered on the epithelial surface. However, when incubated with antiserum, mucus almost completely filled the lumen and coated the fecal pellets. Bacteria in these stabilized preparations were limited mainly to the fecal pellets, and there were small numbers scattered in the luminal mucus, but none were observed on the epithelial surface or within the crypts. Latex particles introduced into the lumen with the antiserum or with phosphate-buffered saline showed the same distribution as the bacteria. These findings are at variance with previous reports that organisms occur in abundance in the mucous layer, adjacent to cell surfaces, and inside crypts. Our results suggest that conventional preparation for microscopy without prior stabilization of the mucus in situ may lead to artifactual redistribution of microorganisms and emphasize the importance of mucus in maintaining mucosal-floral homeostasis in the colon.

The distribution of microorganisms in the large bowel has been studied with the aid of scanning electron microscopy, light microscopy, and in vitro culture techniques (4, 13, 15). In the rodent, microorganisms have been observed within the lumen of the gut and mixed with the mucous layer (9). In addition they have been reported attached to the epithelial surface (1, 12), and some believe they extend into the intestinal crypts (12, 16).

A major factor in interpreting studies of this type is the confidence with which one can assume that the preparative procedures have not artifactually distorted the natural relationships between the epithelium, the mucus, and the luminal contents. It is known that conventional preparation of samples for microscopy can cause considerable disintegration, shrinkage, and loss of the highly hydrated mucous layer adjacent to the intestinal epithelium. To circumvent this problem, Rozee et al. (9) developed a technique for the stabilization of the mucous layer using antibodies raised against intestinal mucus. They reported that bacteria and protozoa were present both within the mucous layer and attached to the epithelium of the mouse ileum (9).

We have since extended and modified this method (J. Bollard, M. Vanderwee, G. Smith, C. Tasman-Jones, J. Gavin, and S. Lee, Dig. Dis. Sci., in press) to stabilize mucus relatively undisturbed within segments of rat colon. This paper describes the use of this method to determine the location of bacteria with respect to intestinal contents, the native mucous layer, and the epithelial surface.

MATERIALS AND METHODS

Stabilization of mucus. Antibodies were raised in three New Zealand White rabbits against mucus obtained from the colons of newly killed rats. Fecal matter was removed from six excised rat colons, and mucus was collected by scraping off the surface mucous gel with a glass slide. The pooled mucus was homogenized in 9 volumes of 0.15 M phosphate-buffered saline (PBS) (pH 7.4) and stored at −20°C until used in the immunization schedule described by Hurn and Chantler (2). PBS (0.5 ml) containing 100 μg of protein (6) was first emulsified with 0.5 ml of complete Freund adjuvant with two syringes and a double-hub connector, and then it was injected subcutaneously at eight sites on both sides of the spine of each rabbit. Four weeks later the rabbits were given a booster injection (0.5 ml), following the same procedure. The animals were bled (10 to 20 ml) 7 days after the second injection, and the serum was separated by centrifugation. Antibody activity was confirmed by the tube precipitin test, double diffusion in agarose, and binding to immobilized mucus on cellulose-nitrate membranes (7). A single batch of serum of the highest titer ($1:10^5$) was used in all the experiments. Complement activity in the serum was then removed by incubation at 56°C for 30 min (Bollard et al., in press).

Eleven random-bred, specific-pathogen-free male Wistar rats aged 49 to 56 days (body weight, 180 to 225 g) were raised in polycarbonate cages with wood chips for bedding and fed tap water ad libitum and a standard rodent diet in pellet form (Diet 86; New Zealand Stock Foods Limited, Otahuhu, New Zealand). After fasting overnight they were killed rapidly by cervical dislocation, and a laparatomy was performed. Each colon was dissected out, and two or three segments, each 1 to 2 cm in length, were excised from the middle third of the colon after ligation at one end around a fecal pellet. The lumen of each segment was then gently injected with 0.1 to 0.2 ml of solution without distension, and the other end was ligated.

One segment from each of five animals was injected with antiserum, and another was injected with 0.15 M PBS (pH 7.4). All specimens were incubated for 1 h at 4°C before the ligatures were removed, and the segments were fixed by immersion in 2.5% phosphate-buffered glutaraldehyde for 16 h.

To investigate whether conventional histological preparation could induce artifacts in the spatial distribution of microparticles, latex particles (0.8-μm average diameter; Difco Laboratories) in either PBS or antiserum were care-

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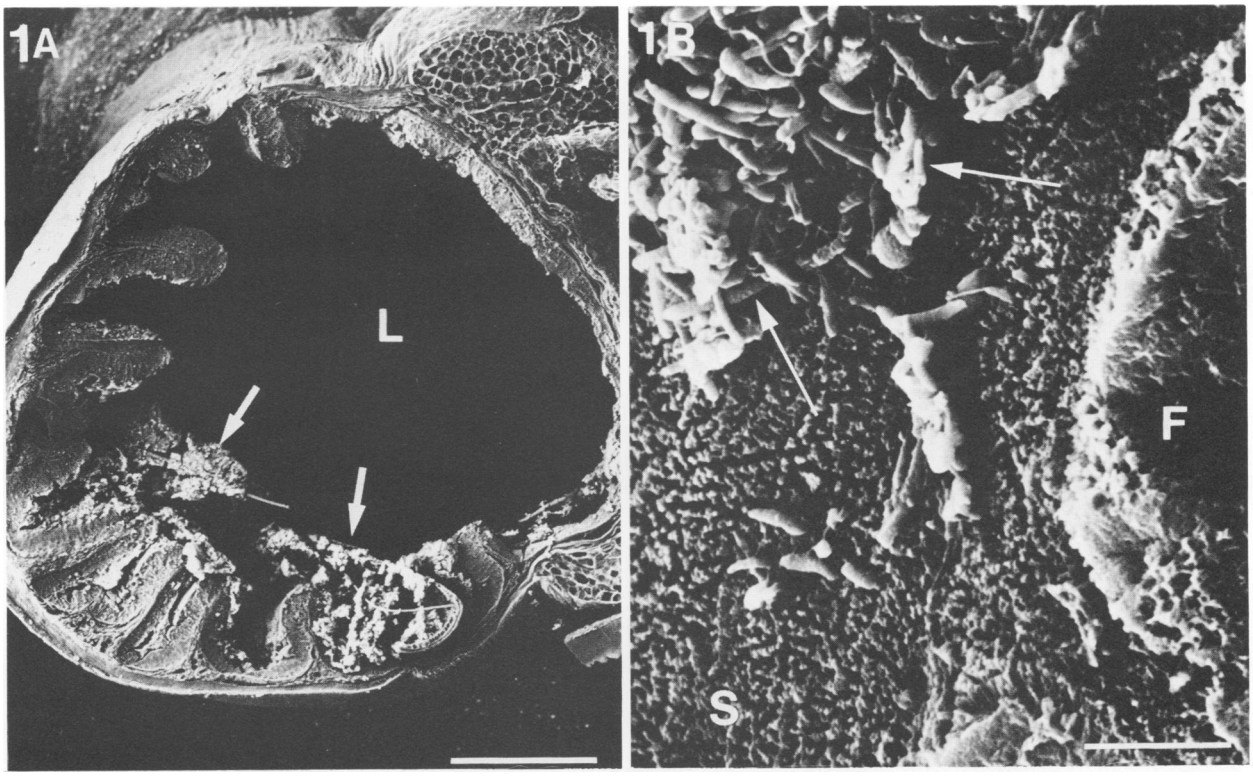

FIG. 1. Transverse fracture planes across a colon incubated with PBS. (A) A largely empty lumen (L) with mucus and gut content (arrows) condensed onto the epithelial surface (×21; bar = 900 μm). (B) Junction between the fracture face (F) and the epithelial cell surface (S) on which numerous bacteria (arrows) are scattered (×3,200; bar = 6 μm).

fully injected into the lumen of segments of colon from six rats. These specimens were then incubated for 1 h and fixed.

In addition, we studied the possibility that the process of incubation with antiserum within the closed segments might itself give rise to artifacts, for example, by stimulating or allowing the secretion of mucus in addition to the mucous layer originally present. With rats under halothane anesthesia, a thoracotomy was performed on two animals. The aorta and right atrium were cannulated (5) and then perfused with 2.5% glutaraldehyde through the aortic cannula until the right atrial return was clear. Colon segments fixed in situ in this way were then incubated with antiserum or PBS.

Scanning electron microscopy. After glutaraldehyde fixation, all 22 samples were washed in 0.1 M phosphate buffer and dehydrated in increasing concentrations of tertiary butanol (17). Special care was taken in changing solutions to minimize fluid shear which might disrupt the mucus.

The segments were then frozen in liquid Freon-12 precooled with liquid nitrogen, immersed in liquid nitrogen (−196°C) until fully quenched, and placed on a precooled brass block. They were then fractured transversely in one or two places with a razor blade precooled in liquid nitrogen and mounted in tongs (10).

The fractured fragments were freeze-dried at −60°C for 16 to 24 h in a tissue drier (Edwards-Pearse EPD 2; Edwards High Vacuum Ltd., Manor Royal, Sussex, England) with phosphorus pentoxide as a vapor trap. When dried, they were mounted on aluminum stubs with copper conducting paint, coated with carbon and then gold by vacuum evaporation, and then examined in an ISI DS-130 research scanning electron microscope fitted with a back-scattered electron detector. The accelerating voltage used was normally 30 kV. One or two complete transverse fracture faces were examined in detail for each sample, and representative photomicrographs were prepared.

Light microscopy. Antiserum-treated and PBS-treated control segments of mid-colon from another five rats were fixed in glutaraldehyde and embedded in paraffin. Transverse sections (5 μm thick) were cut and stained either with PAS alcian blue at pH 1 or 4 to identify sulfated and nonsulfated mucins, respectively (14), or with acridine orange to identify bacteria (3).

RESULTS

Scanning electron microscopy. In segments of colon treated with PBS, the mucous layer was scanty and condensed against the epithelial surface (Fig. 1A). Bacteria were seen incorporated into the strands of mucus, and they also lay on the epithelial surface (Fig. 1B) and in the mouths of some crypts.

However, when incubated with antiserum, the lumen of the colon between fecal pellets was almost completely filled with homogeneous mucus (Fig. 2A) which extended into the intestinal crypts. This material also formed a layer surrounding each fecal pellet (Fig. 3A). Furthermore, bacteria which were observed in abundance within fecal pellets (Fig. 3B) were not observed either within the mucus or on the epithelial surface (Fig. 2B) or within crypts. Similar appearances were seen in material fixed by perfusion before incubation.

Latex particles were identified by their spherical shape and constant known diameter. Those particles which had been introduced into segments incubated with PBS were found intermingled with bacteria, within the condensed strands of mucus, and lying on the surface of the epithelium

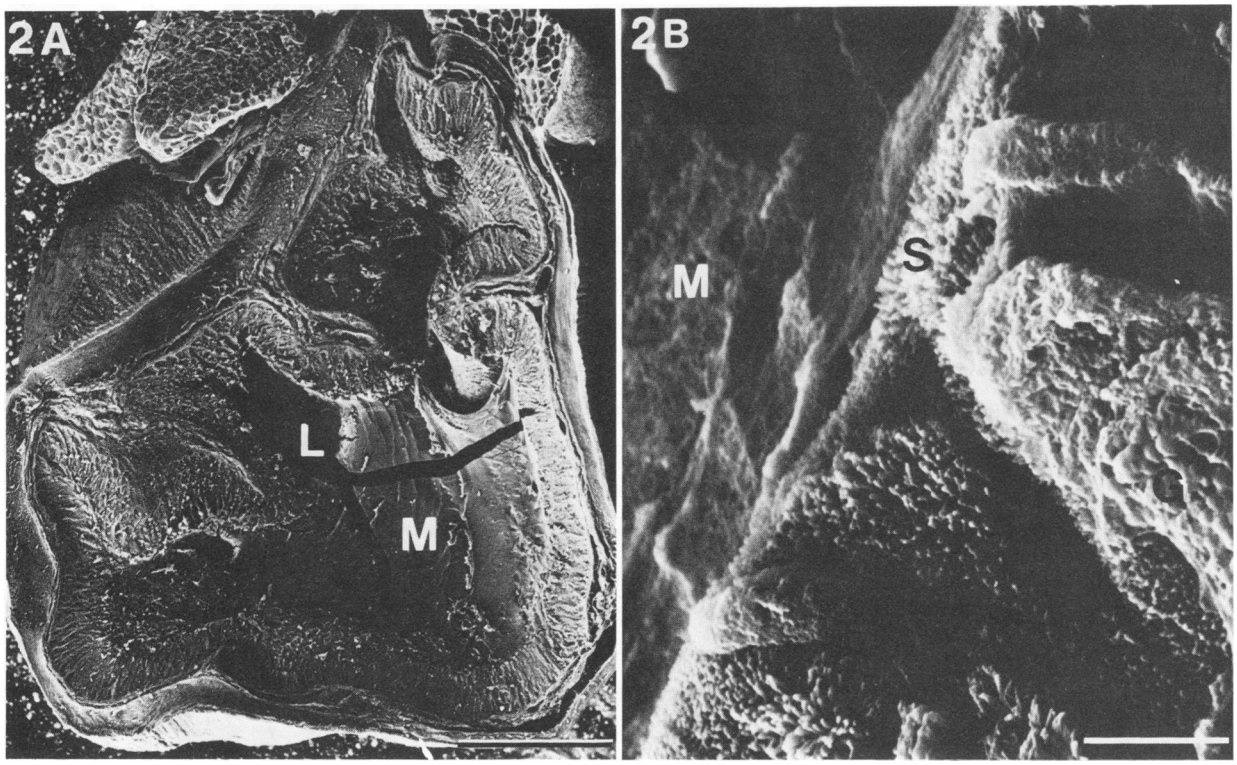

FIG. 2. Transverse fracture planes across a colon incubated with antimucus antibodies. (A) Lumen (L) largely filled with mucus (M) (×25; bar = 800 μm). (B) Fracture plane which passes through a goblet cell (G), across the epithelial surface (S), and through the overlying mucus (M). No bacteria are evident (×3,200; bar = 6 μm).

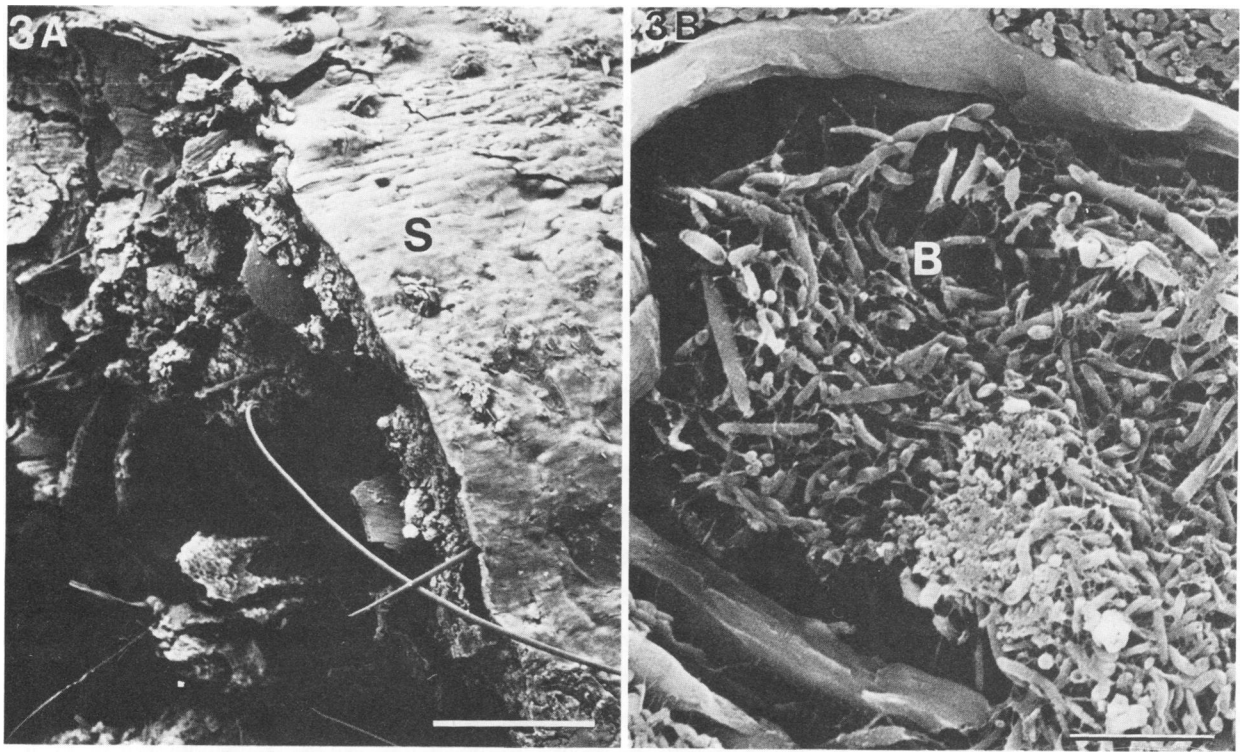

FIG. 3. Freeze-fracture planes through a fecal pellet showing a relatively smooth mucus-covered surface (S) and numerous bacteria (B) within it. (A) ×30; bar = 700 μm; (B) ×2,700; bar = 7 μm.

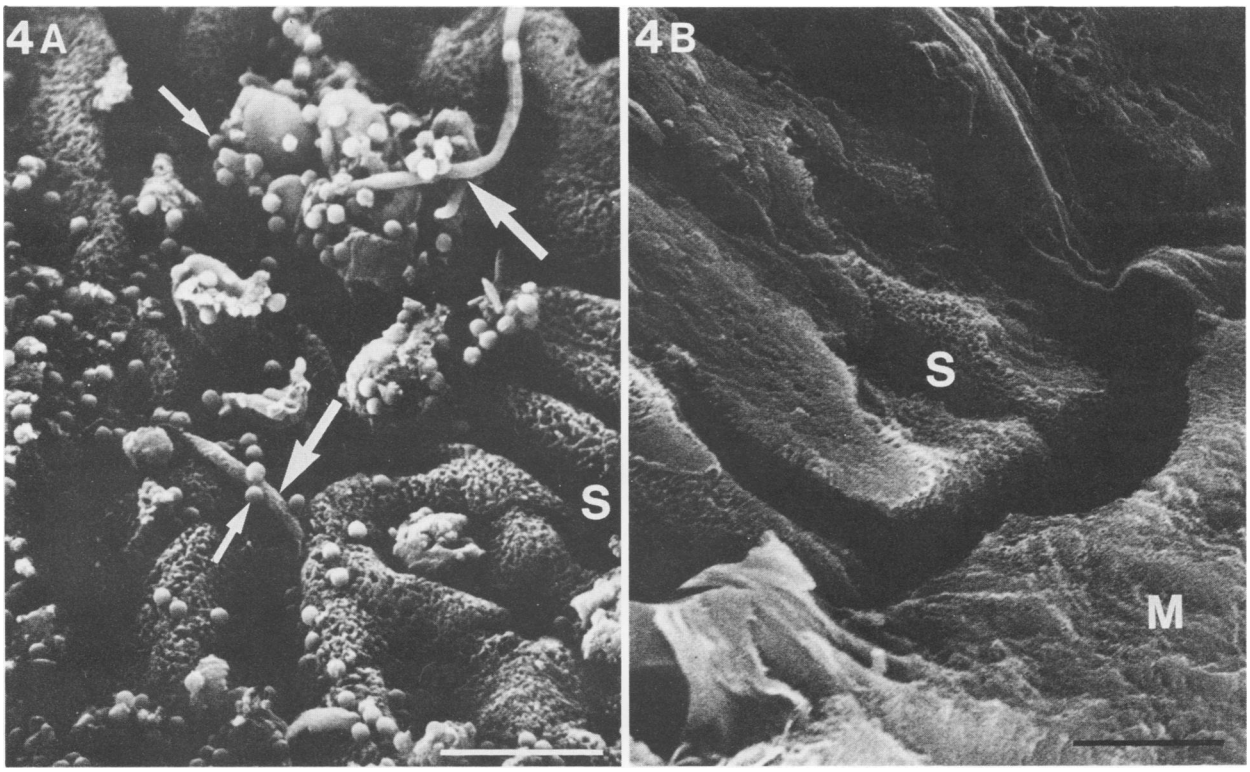

FIG. 4. Freeze-fracture planes passing across the epithelial surface of colon. (A) Colon incubated with PBS and latex particles. The particles (small arrows) lie among microorganisms (large arrows) on the epithelial cell surface (S) (×3,000; bar = 7 μm). (B) Colon incubated with antiserum containing latex particles. The latex particles are not evident either within the mucus (M) or on the epithelial surface (S) (×2,500; bar = 8 μm).

(Fig. 4A). However, when such particles were added with antiserum, they were not observed within the mucus near or on the epithelial cell surface (Fig. 4B).

Light microscopy. In antiserum-treated specimens the mucus present within and adjacent to the goblet cells in the lower third of the crypts contained predominantly unsulfated mucopolysaccharides, but toward the crypt openings the concentration of sulfated mucopolysaccharides increased. Within the lumen the mucus showed a pattern of alternating layers which varied in their degree of sulfation. In samples not treated with antiserum, only scattered discontinuous regions stained positively for mucus which showed no regional variation or lamellar pattern of sulfation.

Bacteria stained bright orange with acridine orange and were present in very large numbers in the fecal pellets. In antiserum-treated samples there were a few isolated bacteria scattered in the luminal mucus, but none were observed within the crypts or at the epithelial surface. However, in specimens not treated with antiserum, the bacteria had a distribution similar to that observed by scanning electron microscopy.

DISCUSSION

This study confirmed the risk of artifactual redistribution of bowel content during preparation for morphological examination and, by using a method which stabilizes mucus in situ, demonstrated that in the mid-colon of the rat bacteria seldom come in contact with the epithelial surface. The distribution of the flora in this region has not been extensively studied. Only Phillips et al. (8) appear to have compared the proximal and mid-colon in this respect, and they observed distinct differences in the organisms present in mucosal scrapings from these two regions.

The difference in the apparent location of microorganisms in the colon depending on whether or not the mucus was stabilized to preserve its distribution in vivo was dramatic. When samples were prepared by conventional methods without stabilizing the mucus, we obtained results similar to those reported by others (11, 12, 16). However, comparison of similar material with samples treated with antiserum raised against whole mucus confirmed that the dehydration stages in routine methods of preparation cause massive shrinkage and disintegration of the highly hydrated mucus (Bollard et al., in press). The use of closed segments of colon and freeze-fracture further minimized the risk of mechanical disruption of relationships between the colon and its contents. It is conceivable that the presence of serum or PBS in the lumen for 1 h before fixation facilitated bacterial translocation, but in all samples in which the mucus was stabilized, bacteria were never observed within the crypts or close to the epithelium. The artifactual redistribution in conventionally prepared samples was confirmed by the experiments with latex particles of similar dimensions to those of bacteria.

When stabilized by antibody cross-linking (Bollard et al., in press), the mucus was preserved as a structurally homogeneous mass coating the epithelium, surrounding the fecal pellets, and filling a substantial proportion of the lumen between pellets. Perfusion fixation of the mucosa in vivo before incubation eliminated the possibility that this considerable volume of mucus arose by its continued secretion during incubation. Freeze-fracture planes through this mu-

cus were glasslike and did not show discontinuities consistent with a significant bacterial content. The sparsity of bacteria within the mucus was confirmed by light microscopy.

Previous investigators of the relationship between the intestinal flora and mucosa have concluded, on the basis of conventional methods, that in the rodent colon bacteria are distributed within the lumen and extended to within close proximity (9) or are attached to (1, 12) the epithelial cells. The results of the present study open these previous interpretations to question, although we acknowledge that both the distribution and the nature of the bacterial flora do change along the length of the gut (8).

Our observations indicate that although bacteria are present in abundance in the mid-colon, they are usually separated from mucosal epithelial cells by a continuous blanket of mucus which can act as a diffusion barrier (13a). It is possible that this mucous layer serves as a barrier to bacterial attachment to the mucosa. This is consistent with the belief that there is a strong correlation between the pathogenicity of various strains of bacteria and their ability to attach to the host mucosal cells. This hypothesis is also in keeping with the protective function of mucus and with the concept that alterations in the quality and quantity of mucus can alter the relationship between the luminal flora and the mucosa, and lead to the development of disease.

ACKNOWLEDGMENT

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