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Microflora of the Gastrointestinal Tract

A Review

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1. Introduction

The mucosal surface of the human gastrointestinal (GI) tract is about 200–300 m^2 and is colonized by 10^{13-14} bacteria of 400 different species and subspecies. Savage *(1)* has defined and categorized the gastrointestinal microflora into two types, autochthonous flora (indigenous flora) and allochthonous flora (transient flora). Autochthonous microorganisms colonize particular habitats, i.e., physical spaces in the GI tract, whereas allochthonous microorganisms cannot colonize particular habitats except under abnormal conditions. Most pathogens are allochthonous microorganisms; nevertheless, some pathogens can be autochthonous to the ecosystem and normally live in harmony with the host, except when the system is disturbed *(2)*.

The prevalence of bacteria in different parts of the GI tract appears to be dependent on several factors, such as pH, peristalsis, redox potential, bacterial adhesion, bacterial cooperation, mucin secretion, nutrient availability, diet, and bacterial antagonism. Because of the low pH of the stomach and the relatively swift peristalsis through the stomach and the small bowel, the stomach and the upper two-thirds of the small intestine (duodenum and jejunum) contain only low numbers of microorganisms, which range from 10^3 to 10^4 bacteria/mL of the gastric or intestinal contents, mainly acid-tolerant lactobacilli and streptococci. In the distal small intestine (ileum), the microflora begin to resemble those of the colon, with around 10^7–10^8 bacteria/mL of the intestinal contents. With decreased peristalsis, acidity, and lower oxidation-reduction potentials, the ileum maintains a more diverse microflora and a higher bacterial population *(3)*. Probably because of slow intestinal motility and very low oxidation-reduction potentials, the colon is the primary site of microbial colonization in humans. The colon harbors tremendous numbers and species of bacteria. However, 99.9% of colonic microflora are obligate anaerobes.

2. Effects of Gastrointestinal Microflora

The most important beneficial effect of the indigenous microflora is to make it more difficult for exogenous pathogenic bacteria to colonize the GI tract and cause disease, a phenomenon known as colonization resistance *(4)*. Indigenous microflora create this barrier effect by occupying available habitats (adhesion sites) at the mucosal level, competing for metabolic substrates and the production of regulatory factors such as short-chain fatty acids and bacteriocins.

On the other hand, indigenous microflora may have potentially deleterious influences on the host's health. There are strong links among dietary factors, the metabolic activities of the indigenous GI microflora, and bowel cancer *(5)*. In fact, it is well known that indigenous GI bacteria transform certain dietary substances into precarcinogens or carcinogens.

Colonization of bacteria is a prerequisite to affect the host, and the process of colonization is directly dependent on the ability of a resident to adhere to the substratum.

3. Adhesion

Adhesion or adherence is defined as the measurable union between a bacterium and a substratum. A bacterium is said to have adhered to a substratum when energy is required to separate the bacterium from the substratum *(6)*.

Adhesion of bacteria to intestinal mucosa is often recognized as a prerequisite for colonization of the human GI tract. Therefore, adhesion properties are important for both pathogenic bacteria and bacteria belonging to the normal human gut microflora.

The study of adhesion will give us better insight into the nature of the interaction between bacteria and cell surfaces. One particular goal in adhesion studies is to be able to inhibit or promote adhesion according to a particular need. Detailed knowledge of the characteristics of adhesins and receptors will provide new approaches to the prevention of serious bacterial infections by interfering with the adhesion process. For example, the bacterial lectin that serves as an adhesin (i.e., carbohydrate-binding adhesin) can be inhibited either by antibodies or by relatively high concentrations of soluble carbohydrates specific for the lectin *(7)*. Moreover, one can screen and characterize certain types of probiotic to exclude specific pathogens or groups of pathogens based on adhesin and receptor properties *(8)*.

3.1. Animal Cell Surface

All animal cell membranes have common compositional and organizational features. Receptors for bacterial adhesions are found in all three classes of membrane constituents, namely, integral, peripheral, and cell surface coat components.

Integral membrane constituents include glycolipids and glycoproteins. Chemically, the glycolipids are either neutral or acidic. The acidic glycolipids contain sialic acids (i.e., gangliosides), which contribute significantly to the net negative charge of the animal cell surface. Virtually all proteins of animal membranes are glycosylated and are similar to soluble glycoproteins *(9)*. There are no significant differences in the overall amino acid composition and carbohydrate content. The monosaccharide constituents usually include the hexoses D-galactose and D-mannose and the methylpentose

L-fuctose, *N*-acetylhexosamines, and the sialic acids. In addition, two types of carbohydrate-peptide linkages are common to both groups of glycoproteins: the *N*-glycosyl bond between *N*-acetyl-D-glucosamine and asparagines (*N*-acetyl-D-glucosaminyl-asparagine) and the *O*-glycosidic bond between *N*-acetyl-D-galactosamine and a serine or threonine of the protein polypeptide backbone.

The peripheral proteins and glycoproteins are anchored to the surface of the membrane by weak ionic interactions or by hydrogen bonding with integral constituents of the cell membranes. Fibronectin is a component of peripheral membrane constituents. The structure of fibronectin is characterized by the presence of several distinct domains with binding sites for specific ligands, such as heparan sulfate, collagen, and hyaluronic acid *(10)*. Numerous bacteria are able to bind to membrane-associated fibronectin.

The cell coat is a substantial layer of carbohydrate-containing materials of variable thickness outside but in close association with the plasma membrane. In healthy human beings, mucosal cells of the respiratory, GI, and urinary tracts are coated with a layer rich in highly sialylated, high molecular weight glycoproteins known as mucins. For a bacterium to colonize tissues, it must either bind to or penetrate the cell coat.

3.2. Bacterial Cell Surfaces

The surface composition of bacteria may vary with the stage of the bacteria, the medium composition, and the presence of antimicrobial agents, all of which may influence adhesin function directly or indirectly. In most cases, the bacterial adhesions are assembled and must dock or anchor on the bacterial surface before they can participate in adhesive processes.

There are four major cell "compartments" that serve to anchor adhesins onto the bacterial surface. Three compartments, namely, the cytoplasmic membrane, peptidoglycan, and the S layer, are found in both Gram-positive and Gram-negative bacteria, and one compartment, the outer membrane, is found only in Gram-negative bacteria.

Bacteria adhere to host cell targets by means of adhesins, which are proteins that recognize a defined carbohydrate sequence on host cell glycoproteins, glycolipids, or, less often, a defined protein structure. In Gram-negative bacteria, adhesins are often located on different types of appendages protruding from the bacterial surface: fimbrial, fibrillar, or curli *(11,12)*. In Gram-positive bacteria, adhesins are usually located in the cell wall or surface coat, although fimbrial structures have been demonstrated on vaginal lactobacilli *(13)*. A bacterium may carry one or several adhesins. The synthesis of fimbriae and adhesins is switched on and off by the bacteria, depending on the environmental conditions, a process called phase variation.

In general, bacterial adhesion to substrata involves two types of mechanisms, specific adhesin-receptor interactions and nonspecific interactions.

3.3. Specific Adhesion

Specific adhesion was defined by Ofek and Doyle *(6)* as an association between the bacterium and substratum that requires rigid stereochemical constraints. Specific adhesion may require hydrogen bond formation, ion-ion pairing, or the hydrophobic effect. In its simplest form, it requires the participation of two factors: a receptor and an adhesin.

A typical example of stereospecific interaction in bacterial adhesion is that between a lectin (carbohydrate-binding lectin adhesin) and a carbohydrate (on the intestinal surface) *(14)*. A well-studied example is the type I fimbriae of *Escherichia coli*, which recognize D-mannose as the receptor site on the host mucosal surface. These adhesin-receptor interactions will lead to irreversible adhesion and may be blocked by specific carbohydrate analogs of the receptor site at the host cell surface *(15)*. The adhesin molecules can be regulated by the bacteria under different condition. Many bacteria have evolved protein adhesins whose specificity limits their choice of econiches. The selective expression of a specific adhesion in response to given environmental demands is an important mechanism that allows the organism to proliferate and survive *(16)*.

The facultative anaerobes have lower adhesion to Caco-2 cells after incubation in the anaerobic condition compared with the aerobic condition. It has been shown that in the aerobic condition more adhesins are expressed than in the anaerobic condition (**Fig. 1**). A recent study has shown that *Bacteroides thetaiotaomicron* can induce matrilysin production is mammalian cells, but it was not detected in germ-free mice. Matrilysin is known for its function in the repair of epithelium and adhesion modification *(17)*.

3.3.1. Fucose and Mannose As Modulators for Adhesion

Fucose suppressed the adhesion of lactobacilli but enhanced the adhesion of the other GI bacteria on Caco-2 cells (**Fig. 2**).

We also found that the effect of fucose on the adhesion of *Lactobacillus casei shirota* on Caco-2 was owing to a decrease in the number of adhesions on the bacterial surface but an increase in the affinity of the adhesions for the receptors (data not shown). The effect of fucose on *E. coli* 11775 was owing to an increase in the number of adhesins on the bacterial cell surface. An earlier study demonstrated that the occurrence of *B. thetaiotaomicron (18)* induces the production of host GDP-L-fucose (β-D-galactoside 2-α-L-fucosyltransferase) in the ileum of germ-free mice. Although the effect and roles of fucose on intestinal bacteria are not clear *(19)*, its effect on adhesion is obvious.

Mannose strongly enhanced (increase of 220%) the adhesion of *Bacteroides* organisms to Caco-2 cells and had no effect on lactobacilli (**Fig. 3**). The study suggests that free fucose and mannose on the intestinal mucosal surface could serve as modulators of intercellular communication between the bacteria and the host for regulation of the bacteria population on the intestinal surface.

In germ-free mice, preculturing both the *E. coli* 11775 and *B. fragilis* in mannose altered their adhesion on the small intestinal surface but not on the colon surface (Lee et al., unpublished data). This observation suggests that the properties of bacterial surfaces adhesins are different, the adhesins to small intestine surface receptors being mannose-dependent, and those for the colon being mannose-independent. It also may be possible that the free mannose had bound to the adhesins, preventing them from binding onto the small intestinal surface receptors, and thus resulting in lower concentrations of adherent mannose-cultured *E. coli.* Klemm and Schembri *(20)* classify the
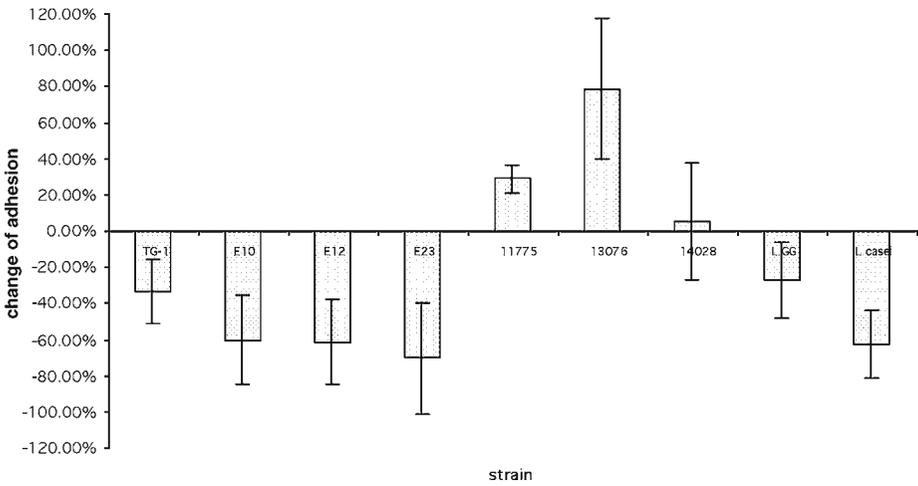
Fig. 1. Percent changes in adhesion to Caco-2 cells of *Lactobacillus*, *E. coli*, and *Salmonella* organisms incubated in the anaerobic compared with aerobic condition. TG1, *E. coli*; E10, *S. typhimurium*; E12, *S. typhimurium*; E23, *S. bellurup*; 11775, *E. coli*; 13076, *S. choleraesuis* subsp. *choleraesuis* serotype *enteritidis*; 14028, *S. typhimurium*; LGG, *L. rhamnosus*; *L. casei*, *L. casei*.
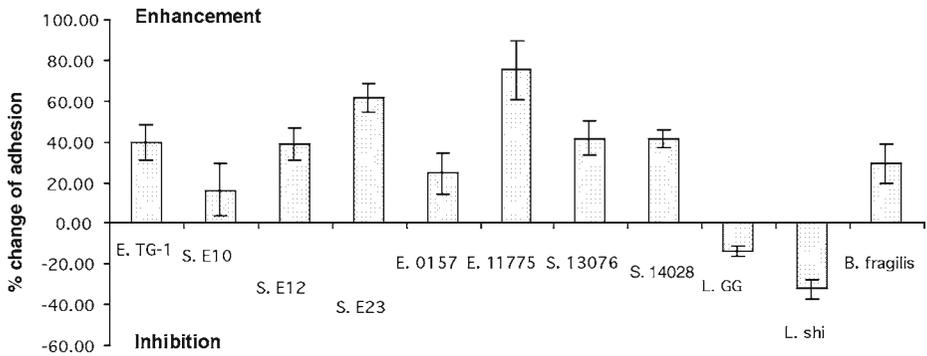
Fig. 2. Percent changes in adhesion to Caco-2 cells of lactobacilli and GI bacteria cultured with fucose (0.5% w/v) in media. ETG1, *Escherichia coli*; SE10, *Salmonella typhimurium*; SE12, *S. typhimurium*; SE23, *S. bellurup*; EO157, *E. coli*; E11775, *E. coli*; S13076, *S. choleraesuis* subsp. *choleraesuis* serotype *enteritidis*, S14028, *S. typhimurium*; LGG, *L. rhamnosus*; *L. shi*, *L. casei*; *B. fragilis*, *Bacteroides fragilis*.
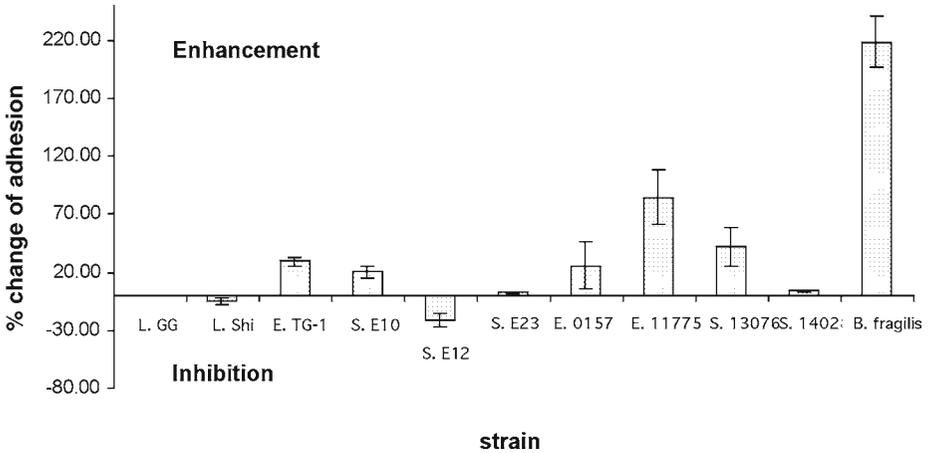
Fig. 3. Percent changes in adhesion to Caco-2 cells of lactobacilli and GI bacteria cultured with mannose (0.5% w/v). LGG, *Lactobacillus rhamnosus*; *L. shi*, *L. casei*; ETG1, *Escherichia coli*; SE10, *Salmonella typhimurium*; SE12, *S. typhimurium*; SE23, *S. bellurup*; EO157, *E. coli*; E11775, *E. coli*; S13076, *S. choleraesuis* subsp. *choleraesuis* serotype *enteritidis*; S14028, *S. typhimurium*; *B. fragilis*, *Bacteroides fragilis*.

adhesins of *E. coli* into two groups: mannose-sensitive (which involved FimH of type 1 fimbriae) and mannose-resistant. Our study found that the mannose sensitivity of adhesion depends on the site of the GI tract.

3.4. Nonspecific Adhesion

An association between a bacterium and a substratum that involves the same forces does not require a precise stereochemical fit. Nonspecific interaction basically involves the physicochemical forces, i.e., van der Waals and electrostatic forces, hydrogen bonding, and hydrophobic bonding.

4. In Vitro Models for the Study of Adhesion

Adhesion is difficult to study in vivo, and therefore many in vitro methods have been developed.

4.1. Human Intestinal Cell Lines

Cultured cell lines from human intestinal origin are commonly used to study bacterial adhesion, because the cell lines mimic the epithelium of the intestine. Caco-2 and HT-29 are the most used cell lines; they are known to display a typical enterocytic differentiation. Although the cell lines originate from the colon, the cells are able to differentiate into enterocytes *(21)*. The differentiation and polarization of Caco-2 cells is spontaneous, without the requirement of inducers, but the differentiation of the HT-29 cell line only occurs by replacing the glucose in the medium with galactose *(21)*. Another good choice of a human intestinal cell line is HT-29-MTX, a stable

mucus-secreting subpopulation of HT-29 cells created by adapting the cell line to methotrexate *(22)*. The time-course for this cell line to differentiate is much longer, 30 d. These valuable tools share the morphological and physiological characteristics of normal enterocytes and permit studies dealing with adhesion of enteropathogens.

4.2. Human Intestinal Mucus

In healthy human beings, the intestinal epithelial surface is covered by a layer of mucus, comprised mainly of mucin (mucus glycoprotein). Mucus glycoproteins are synthesized and secreted from the salivary glands, esophagus, stomach, small and large intestine, gallbladder, and pancreatic ducts.

The mucins are composed of glycoprotein monomers linked through disulfide bridges. Thus the mucins are characterized by their high molecular weight and consist of a number of carbohydrate side chains, composed of *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, fucose, and sialic acid, attached to a protein core *(23)*. The presence of sialic acids and esters of sulfate makes the mucus more viscous and less susceptible to bacterial attack. There is a core region containing *N*-acetylgalactosamine linking the oligosaccharide side chain to the protein core; a backbone region often branched, of repeating D-galactose and *N*-acetylglucosamine; and a peripheral region at the nonreducing end where the terminal sugar is responsible for the antigenicity of the mucin *(24)*. The ratio of mucin constituent protein to carbohydrate is about 1:4 by weight.

Woods and coworkers *(25)* found that pili mediated the attachment of *Pseudomanas aeruginosae* to buccal epithelial cells. Pili have protein subunits, with a characteristic *N*-methyl-phenylalanine (NMetPhe) residue, that are common to *Vibrio cholera*, *Neisseria gonorrhoea*, and *B. nodosus*. The same authors suggested that outer menbrane proteins and flagellae account for non-pilus-mediated adhesion, although specific receptors have not been defined.

Using mucin as in vitro model, He and coworkers *(26)* reported that the mucosal adhesive property of bifidobacteria was reduced with the aging of the host, and it was suggested that reduced adhesive ability causes the low levels of bifidobacteria found in aging people. An earlier study showed that infant-type bacteria adhered better to adult mucin than to infant mucin and that adult-type bacteria adhered better to infant mucin than to adult mucin. This finding suggested that there are many other factors such as immunity that control the intestinal flora *(27)*.

Immobilized human intestinal mucus glycoproteins have been used as substrata for lactobacilli and GI pathogen adherence *(28)*. Both mucus glycoproteins isolated from feces and ileostomy glycoproteins from ileostoma patients have been used as a model for intestinal mucus and typical small intestinal mucus, respectively *(29,30)*.

5. In Vivo Model for Study of Bacterial Interaction

The most widely used models are germ-free mice and antibiotic-treated mice. Although the bacterial species in the human GI tract are different from those in mice, it is likely that the principles involved in bacterial competition within the mouse gut hold for interactions in the human gut as well. Freter et al. *(31)* established a con-

tinuous-flow (CF) culture, i.e., inoculating the mouse flora into a CF culture within an anaerobic chamber, and maintaining the entire ecosystem of the culture in equilibrium for months. The CF culture was composed of anaerobic species at about the same proportions as in the conventional mouse. The CF culture model has been used to investigate the mechanisms of interaction between *Clostridium difficile* and the colonic flora *(32)*. Similar models have been used to study human flora *(33)*. Minekus et al. *(34)* established a computer-controlled system to simulate conditions in the large intestine. This system showed a stable microflora population after inoculation with human fecal samples, but it could not mimic colonization in the intestine.

6. Interaction Between the GI Tract and Lactobacilli

In vitro studies have shown that lactobacilli were able to compete with many pathogenic bacteria for adhesion. (**Fig. 4**),

In addition, lactobacilli produce antagonistic substances including organic acids such as lactic acid, low molecular weight antimicrobial substances such as reuterin, and high molecular weight bacteriocins. All these substances are able to suppress growth and interfere with the adhesion of pathogenic strains of bacteria. Antimicrobial activity against *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) adhesion and invasion of the Caco-2 cell line by strains of *Lactobacillus* GG, *L. acidophilus* LB, and *L. acidophilus* LA1 was documented *(35)*.

Heinemann and co-workers *(36)* noted that a surface binding protein from *L. fermentum* RC-14 inhibited the adhesion of *Enterococcus faecalis* 1131. Interestingly, Mack and co-workers *(37)* found that the ability of *L. plantarum* 299v to inhibit adherence of pathogenic *E. coli* to HT-29 cells was mediated through their ability to increase expression of MUC2 and MUC3 intestinal mucins.

Other than the adhesion-inhibiting activity mentioned above, it was also found that *Lactobacillus* whole cells and cell wall fragments were able to exclude pathogens competitively *(38)*. In addition, Coconnier et al. *(39)* reported that *L. acidophilus* could prevent, by steric hindrance, the attachment of enterogenic *E. coli* to enterocyte-like Caco-2 cells. It was suggested that the strong antiadhesion activity of *L. crispatus* JCM 8779 against *E. faecalis* appears to be a combined effect of both bactericidal activity and competition for attachment site. Osset et al. *(40)* showed that strains of *Lactobacillus* (especially hemagglutination groups III) were able to block (by exclusion, competition, and displacement) uropathogen adherence, although this was strain-dependent, indicating that both lactobacilli and uropathogens compete for receptors on vaginal epithelial cells. Using mucins extracted from human feces as a model for intestinal mucus, the adhesion of *S. typhimurium* was significantly inhibited by *L. johnsonii* LJ1 and *L. casei shirota* *(41)*. Lactobacilli have been demonstrated to possess surface adhesins similar to those on bacterial pathogens and are thus compete for the specific receptors on the mucosal surface. For example, Neeser et al. *(42)* reported that *L. johnsonii* La1 shares carbohydrate binding specificities with several enteropathogenic bacteria, and Mukai et al. *(43)* proposed that *L. reuteri* strains share glycolipid specificity with *Helicobacter pylori*. These findings confirm that the bacteria itself can potentially block the adhesion of pathogens to the intestinal mucosal surface.
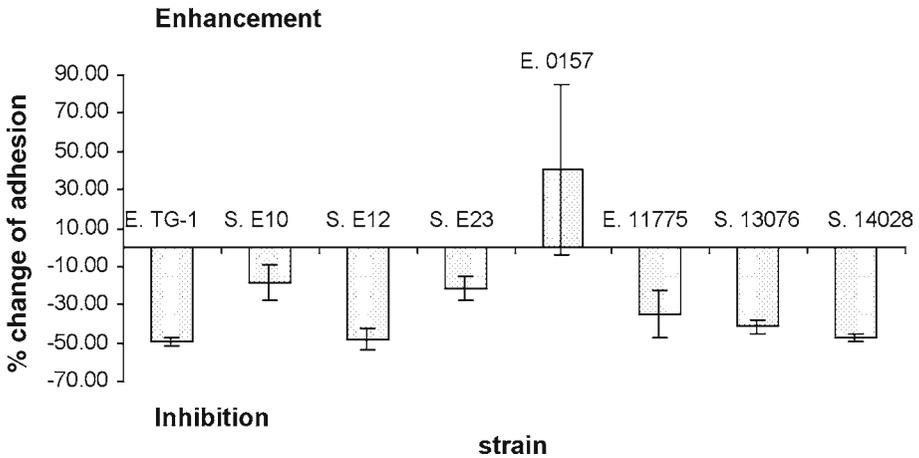
Fig. 4. Competition for adhesion between *Lactobacillus rhamnosus* GG and GI bacteria on Caco-2 cells. ETG1, *Escherichia coli*; SE10, *Salmonella typhimurium*; SE12, *S. typhimurium*; SE23, *S. bellurup*; EO157, *E. coli*; E11775, *E. coli*; S13076, *S. choleraesuis* subsp. *choleraesuis* serotype *enteritidis*; S14028, *S. typhimurium*.

Lactobacilli have been shown to inhibit bladder cancer growth in vivo *(44)* and to prevent recurrence of human superficial bladder cancer *(45)*. In recent studies lactobacilli have been shown to induce production of interleukin-6 (IL-6), granulolyte macrophage colony-stimulating factor, and IL-8, which are cytokines that mediate potent immune responses *(46)*. Our study shows that *L. casei shirota* can help in the recovery of the total population of microbes in ampicillin-treated mice. The total population in the duodenum and ileum increases steadily 1 d after introduction of *L. casei shirota*. The pathogen *E. coli* O157 did not have this property, however.

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