APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2000, p. 3692–3697 Vol. 66, No. 9
0099-2240/00/$04.00+0

Quantitative Approach in the Study of Adhesion of Lactic Acid Bacteria to Intestinal Cells and Their Competition with Enterobacteria

Y. K. LEE,[1]* C. Y. LIM,[1] W. L. TENG,[1] A. C. OUWEHAND,[2] E. M. TUOMOLA,[3] AND S. SALMINEN[2]

Department of Microbiology, Faculty of Medicine, National University of Singapore, Singapore 117597, Republic of Singapore,[1] and Department of Biochemistry and Food Chemistry, University of Turku, FIN-20014 Turku,[2] and Novatreat Inc., FIN-20700 Turku,[3] Finland

Received 10 May 2000/Accepted 20 June 2000

To describe the phenomena of bacterial adhesion to intestinal cells and the competition for adhesion between bacteria, mathematical equations based on a simple dissociation process involving a finite number of bacterial receptors on intestinal cell surface were developed. The equations allow the estimation of the maximum number of *Lactobacillus* sp. and *Escherichia coli* cells that can adhere to Caco-2 cells and intestinal mucus; they also characterize the affinity of the bacteria to Caco-2 cells and intestinal and fecal mucus and the theoretical adhesion ratio of two bacteria present in a mixed suspension. The competition for adhesion between *Lactobacillus rhamnosus* GG and *E. coli* TG1 appeared to follow the proposed kinetics, whereas the competition between *Lactobacillus casei* Shirota and *E. coli* TG1 may involve multiple adhesion sites or a soluble factor in the culture medium of the former. The displacement of the adhered *Lactobacillus* by *E. coli* TG1 seemed to be a rapid process, whereas the displacement of *E. coli* TG1 by the *Lactobacillus* took more than an hour.

Probiotics are viable bacterial cell preparations or components of bacterial cells that have beneficial effects on the health and well being of the host (9, 17). Many of the probiotic bacteria are lactic acid bacteria and are useful in the treatment of dysfunctions with disturb intestinal microflora and abnormal gut permeability (10). Successful probiotic bacteria are usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa (1, 11, 19, 20). Studies have also suggested that adhesive probiotic bacteria could prevent the attachment of pathogens, such as coliform bacteria and clostridia, and stimulate their removal from the infected intestinal tract (1, 11, 19, 20).

Laboratory models using human intestinal cell lines such as Caco-2 (2, 5, 8, 15, 22) and intestinal mucus (13) have been developed to study the adhesion of probiotic lactic acid bacteria and their competitive exclusion of pathogenic bacteria. In this study, a quantitative approach is proposed for the design of experiments and interpretation of data in laboratory studies using cell line and mucus models. This approach provides a better insight to the mechanism of competition between probiotic bacteria and pathogens, and thus allows development of more efficient probiotic products.

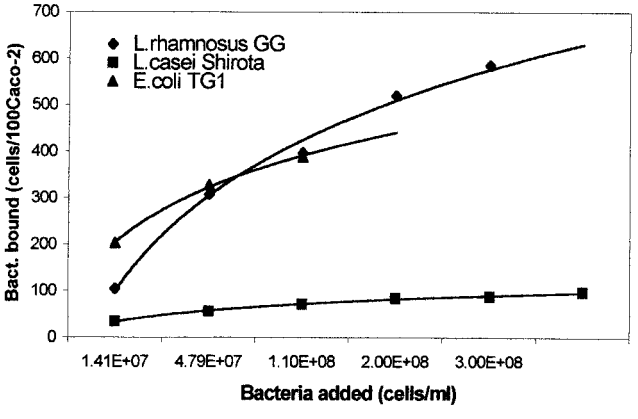

FIG. 1. Adhesion of *L. rhamnosus* GG, *L. casei* Shirota, and *E. coli* TG1 to human intestinal cell line Caco-2, presented as the number of bacteria bound per 100 Caco-2 cells versus the concentration of bacteria added (CFU per milliliter).

* Corresponding author. Mailing address: Department of Microbiology, National University of Singapore, 5 Science Drive 2, Singapore 117597, Republic of Singapore. Phone: 65-874 3284. Fax: 65-776 6872. E-mail: MICLEEYK@NUS.EDU.SG.

MATERIALS AND METHODS

Bacterial strains. Two commercial probiotic strains were used: *Lactobacillus casei* Shirota obtained from Yakult Singapore Pty., Ltd., and *Lactobacillus rhamnosus* GG (ATCC 53103) obtained from the National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, Scotland). Both bacterial strains have clinically demonstrated probiotic properties (9). The bacteria were cultured in MRS broth (BBL Cockeysville, Md.) at 37°C with 5% CO_2 for 18 h before the study. *Escherichia coli* TG1 (Gibson, 1984) was obtained from C. K. Lim (of the Microbiology Department) and it was grown in Luria-Bertani broth (BBL) at 37°C for 18 h before the study. *E. coli* TG1 was chosen for this bacterium has a maximum adhesion to Caco-2 cells which falls between that of *L. casei* Shirota and that of *L. rhamnosus* GG. For the mucus assay (see below) (methyl,1′,2′-3H)

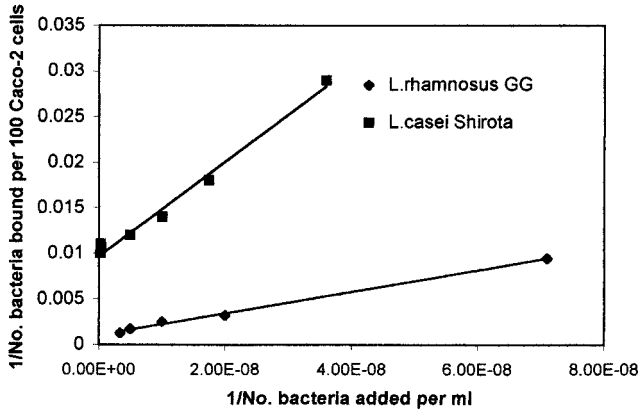

FIG. 2. Double-reciprocal representation of the adhesion of *L. rhamnosus* GG and *L. casei* Shirota to human intestinal cell line Caco-2. The lines indicate the linear fit according to the least-squares method.

TABLE 1. Maximum number of adhered bacterial cells on 100 Caco-2 cells and dissociation constant of the adhesion process for various strains[a]

Strain	e_m (cells/100 Caco-2 cells)	k_x (cells/ml)
L. casei Shirota	137.9	8.62×10^7
L. rhamnosus GG	1,612.9	2.08×10^8
E. coli TG1	500	4.76×10^8

[a] The values were calculated from equation 2 based on the data presented in Fig. 2 and 3.

thymidin was added to the media at a concentration of 10 μl ml^{-1} (117 Ci $mmol^{-1}$) to radiolabel the bacteria.

Intestinal cell culture. The Caco-2 cell culture (7) was used in the adhesion assay. This human colon adenocarcinoma cell line was obtained from the American Type Culture Centre (Manassas, Va.). The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (GIBCO-BRL), containing 25 mM glucose, 20% (vol/vol) heated inactivated fetal calf serum (GIBCO-BRL), and 1% nonessential amino acids (GIBCO-BRL). The cells were grown at 37°C in 5% CO_2. For the adhesion assay, monolayers of Caco-2 cells were prepared in two-chamber slides (Lab-Tek chamber slide; Nunc Inc.) by inoculating 2.8×10^5 viable cells into 2 ml of culture medium. The medium was replaced every two days.

Intestinal mucus. Intestinal mucus was isolated from feces of healthy adult volunteers as described earlier (13). In short, fecal extracts were prepared by homogenizing feces in phosphate-buffered saline (PBS) (pH 7.2) containing protease inhibitors and sodium azide and centrifuging the suspension at 15,000 × *g*. The mucus was isolated from the clear fecal extract by dual ethanol precipitation. The crude mucus was further purified by size exclusion chromatography.

Human ileostomy glycoproteins were a generous gift from J. G. H. Ruseler-van Embden of the Erasmus University, Rotterdam, The Netherlands.

Adhesion assay. (i) On Caco-2 cells. Fifteen-day-postconfluent Caco-2 monolayers were washed twice with 1 ml of sterile PBS before the adhesion assay. One ml of the test bacteria at concentrations between 1×10^5 and 4×10^8 CFU ml^{-1} were added to 1 ml of complete Caco-2 medium. This suspension (2 ml) was added to each chamber of the two-chamber slide and incubated at 37°C, in a 5% CO_2–95% air atmosphere, with gentle rocking. After incubation for 60 min, the monolayers were washed twice with sterile PBS (pH 7.2), fixed with methanol, Gram stained, and examined microscopically. Visual counting of adhered cells was adopted in this study, for it allows the differentiation of the gram-positive *Lactobacillus* and gram-negative *E. coli*. Each adherence assay was conducted in triplicate by two students (Y.Y.L. and W.L.T.), and the number of adherent bacteria was counted on about 1,000 Caco-2 cells, in 60 randomly selected microscopic fields. To stimulate the physiological pH condition of the gastrointestinal tract, all experiments were done at pH 7.

In the study of the competition for adhesion on Caco-2 cells, *Lactobacillus* and *E. coli* were added simultaneously or sequentially to the Caco-2 cultures before counting. In the latter case, free cells of the first bacterium were removed by washing with PBS (pH 7.2) before the second bacterium was added. The lactic acid bacteria have the tendency to form chains and aggregates. It was necessary to disperse the chains and aggregates of the bacterial cells before the adhesion study, to ensure that cells observed under the microscope in the adhesion assay were cells adhered to Caco-2 and ileosotomy glycoprotein surfaces (13).

(ii) On immobilized mucus and ileostomy glycoproteins. The study of adhesion of the microorganisms on mucus and ileostomy glycoproteins was performed as described earlier (13). In short, human intestinal mucus or human ileostomy glycoprotein was passively immobilized in microtiter plate wells. Bacteria were allowed to bind to the mucus or ileostomy glycoproteins at concentrations between 4.4×10^6 and 4.1×10^8 CFU · ml^{-1}. The radioactivity was assessed by liquid scintillation. The relation between the measured radioactivity and the number of bacteria was determined by plate counting.

Theory. In many studies on the adhesion of bacterial cells to intestinal epithelial cells, when the number of bacterial cells adhered to intestinal cells is plotted against the concentration of bacterial culture, a section of a rectangular hyperbola is obtained, as shown in Fig. 1. Such a relationship implies a process of simple dissociation. That is,

$$\text{Bacteria} + \text{Intestinal cell} \underset{k-1}{\overset{k+1}{\longleftrightarrow}} \text{Bacterium-Intestinal cell complex}$$

where $k + 1$ and $k - 1$ represent the dissociation constants for the reaction. The process is similar to the reaction between a substrate and an enzyme that forms a substrate-enzyme complex, without the formation of a product.

The relationship is based on the assumption that the interaction between the bacterial cells and the intestinal cells or mucus remains in equilibrium. This condition should be achieved if the bacterial cells do not penetrate the intestinal cells.

It is also assumed that the concentration of the bacterial culture remained essentially unchanged throughout the study, so that the concentration of the bacterial culture can be considered equal to the initial bacterial concentration. This condition is usually achieved when the total number of bacterial cells is much greater than the number of bacterial cells adhering to the intestinal cells. This is usually the case in most of the adhesion studies, where the concentration of the bacterial cells added is in the range of 10^5 to 10^8 per ml, whereas that of the intestinal cell culture (e.g., Caco-2 cells) is about 10^2 per ml and the number of bacterial cells adhered to the Caco-2 cells is fewer than 10 per cell.

In the equation described above, if x is the concentration of the bacterial culture added, e is the intestinal epithelial cell or mucus concentration, and e_x is the concentration of the bacterium-intestinal cell-mucus complex, then the concentration of free bacterial cells will be $(x - e_x)$.

Because the process is in equilibrium, the dissociation constant for the process (k_x) can be defined as $k_x = (k - 1)/(k + 1) = (x - e_x)x/e_x$. This equation can be rearranged to give an expression for the concentration of the bacterium-intestinal cell-mucus complex, $e_x = e \cdot x/(k_x + x)$. When x is very much larger than k_x, e_x approaches e. The maximum value of e_x obtained when the intestinal cells or mucus is saturated with bacteria as e_m, which may be written as

$$e_x = e_m \cdot x/(k_x + x) \quad (1)$$

When x is equal to k_x, e_x is equal to $e_m/2$; thus, the value of k_x could be experimentally obtained from the value of x, which gives half the maximum e_x (i.e., $e_m/2$).

Equation 1 can be rearranged to give a linear relationship.

$$1/e_x = 1/e_m + k_x/e_m \cdot x \quad (2)$$

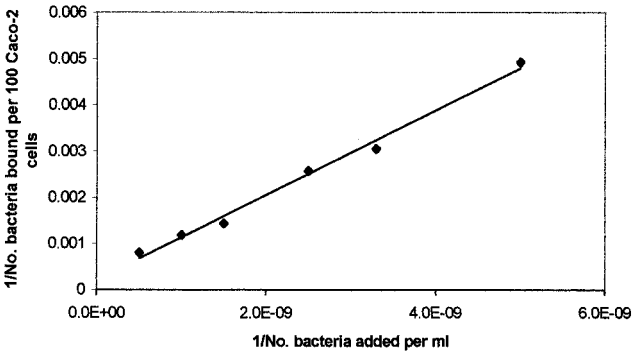

FIG. 3. Double-reciprocal representation of the adhesion of *E. coli* TG1 to human intestinal cell line Caco-2. The lines indicate the linear fit according to the least-squares method.

TABLE 2. Adhesion of bacteria to Caco-2 cells in mixed bacterial suspension[a]

L. rhamnosus added (cells/ml)	Total no. of cells counted			Observed $e_E \pm$ SD	Observed $e_L \pm$ SD	Observed $e_E + e_L$	Observed e_E/e_L	Predicted e_E/e_L
	Adhered *L. rhamnosus*	Adhered *E. coli*	Caco-2					
1×10^8	3,289	420	995	42.21 ± 1.32	330.55 ± 45.40	372.76	0.128	0.166
2×10^8	3,801	281	954	29.45 ± 19.36	398.43 ± 46.64	427.88	0.074	0.071
3×10^8	3,443	130	981	13.25 ± 8.85	350.97 ± 19.08	364.22	0.038	0.041

[a] e_E and e_L number of *E. coli* TG1 and *L. rhamnosus* cells adhered to 100 Caco-2 cells, respectively.

TABLE 3. Adhesion of bacteria to Caco-2 cells in mixed bacterial suspension[a]

L. casei added (cells/ml)	Total no. of cells counted			Observed $e_E \pm$ SD	Observed $e_L \pm$ SD	Observed $e_E + e_L$	Observed e_E/e_L	Predicted e_E/e_L
	Adhered *L. casei*	Adhered *E. coli*	Caco-2					
1×10^8	1,084	147	1,183	12.4 ± 3.82	91.63 ± 10.68	104.03	0.135	1.169
2×10^8	736	68	1,071	6.35 ± 0.35	68.72 ± 7.07	75.07	0.092	1.536
3×10^8	581	36	783	4.60 ± 1.91	74.20 ± 5.41	78.8	0.062	1.806

[a] e_E and e_L, number of *E. coli* TG1 and *L. casei* Shirota cells adhered to 100 Caco-2 cells, respectively.

Hence, a plot of $1/e_x$ against $1/x$ will give a straight line, in which the intercept on the ordinate gives a value of $1/e_m$, and that on the abscissa gives a value of $-1/k_x$.

In the case where two types of bacteria are present in the system and they compete for the same receptors or adhesion sites (through steric hindrance of cells in close vicinity), the competition for adhesion of each of the bacterial types is determined by the affinity of the bacteria to the intestinal cells or the intestinal mucus (k_x) and the concentration of the bacterial culture (x). Thus, the ratio of e_x for bacterium 1 and bacterium 2 can be described as

$$e_{x_1}/e_{x_2} = e_{m_1}/e_{m_2} \cdot x_1/x_2 \cdot (k_{x_2} + x_2)/(k_{x_1} + x_1) \quad (3)$$

Statistics. Differences between treatments were examined for the level of significance by Student's t test after analysis of variance.

RESULTS

Adhesion of bacteria to Caco-2 cells. When the concentration of adhered bacterial cells (cells per 100 Caco-2 cells) was plotted against the concentration of bacterial cells added, a hyperbolic relation was observed for all three of the bacteria tested (Fig. 1).

The plots of the reciprocal of adhered cell concentration versus the reciprocal of the concentration of cells added, for *L. casei* Shirota and *L. rhamnosus* GG are given in Fig. 2, and that for *E. coli* is given in Fig. 3. In all the cases a linear relationship was observed. It follows from equation 2 that the intercept on the ordinate gives the value of the reciprocal of the maximum number of bacterial cells adhered to 100 Caco-2 cells (e_m). The intercept on the abscissa is $-1/k_x$, where k_x is the dissociation constant for the adhesion process. Thus, the values of e_m and k_x for the three bacteria were calculated, and they are summarized in Table 1.

As shown in Table 1, among the three bacteria studied, the maximum number of *L. rhamnosus* GG cells that can adhere to Caco-2 cells is about 10 times that of the *L. casei* Shirota, whereas the maximum adhesion number of *E. coli* TG1 is between those of the two lactobacilli. *E. coli* TG1 was chosen for this study as it would allow us to understand the competition of an enterobacterium with a lactic acid bacterium which has a higher adhesion capacity (i.e., *L. rhamnosus* GG) and with one whose adhesion capacity is lower (i.e., *L. casei* Shirota).

L. casei Shirota's having the lowest k_x implies that it has a higher affinity for adhesion to Caco-2 cells than do *L. rhamnosus* GG and *E. coli* TG1; i.e., adhered *L. casei* Shirota dissociates less easily than the other two bacteria.

Competition between *Lactobacillus* and *E. coli* for adhesion. In this study, various concentrations of a *Lactobacillus* strain (2×10^8 to 6×10^8 cells ml^{-1}) were mixed with an equal volume of *E. coli* (2×10^8 cells ml^{-1}) and then added onto the Caco-2 cells. The final concentration of the respective bacterial strains is thus half of the original concentration. The Gram-stained *Lactobacillus* and *E. coli* adhered on Caco-2 cells could be easily differentiated and counted microscopically. The observed concentrations of the adhered *Lactobacillus* and *E. coli* and the predicted ratio of the two bacteria based on equation 3 are given in Tables 2 and 3.

In the case of *L. rhamnosus* GG, the predicted ratio of *E. coli* and lactobacilli counted on Caco-2 cells (e_E/e_L) is comparable to the observed values (Table 2). In the case of *L. casei* Shirota, the predicted values of e_E/e_L are 1.169 to 1.806 for the three concentrations of *Lactobacillus* added. A value of >1 indicates that the *Lactobacillus* has been excluded by *E. coli* for adhesion to Caco-2 cells. The observed values of e_E/e_L are 8 to 23 times lower than the predicted values, ranging from 0.062 to 0.135.

Exclusion of *E. coli* by adhered lactobacilli. In the study, the lactobacilli (10^8 cells ml^{-1}) were allowed to adhere to Caco-2 cells. Nonadhered *Lactobacillus* cells were removed by PBS, and then the *E. coli* TG1 (10^8 cells ml^{-1}) was added and incubated with Caco-2 cells for 1 h. The respective adhesion number of the two bacteria was counted and is shown in Table 4.

In the case of *L. rhamnosus* GG, the concentration of the *E. coli* TG1 counted (160.56 ± 14.14 cells/100 Caco-2 cells) was not statistically different ($P > 0.05$) compared with *E. coli* when it was added alone and incubated with Caco-2 cells (169.16 ± 15.63 cells/100 Caco-2 cells), whereas in the case of *L. casei* Shirota, the concentration of the *E. coli* TG1 counted (122.26 ± 15.66 cells/100 Caco-2 cells) was significantly lower ($P < 0.05$) than that of *E. coli* (169.16 ± 15.63 cells/100 Caco-2 cells). However, in both of the studies involving *L. rhamnosus* GG (62.36 ± 11.10 cells/100 Caco-2 cells) and *L. casei* Shirota (53.95 ± 2.82 cells/100 Caco-2 cells), the counts of the lactobacilli were much lower than those when *L. rhamnosus* (397.7 ± 53.2 cells/100 Caco-2 cells) and *L. casei* (78.94 ± 9.60) alone were incubated with Caco-2 cells. The values of e_E/e_L were greater than 1; i.e., there were more *E. coli* cells than lactobacilli.

TABLE 4. Results of the exclusion study in which *Lactobacillus* cells were allowed to adhere to Caco-2 cells before *E. coli* was added[a]

Strain	Total no. of cells counted			Observed $e_E \pm$ SD	Observed $e_L \pm$ SD	Observed $e_E + e_L$	Observed e_E/e_L
	Adhered *Lactobacillus*	Adhered *E. coli*	Caco-2				
L. rhamnosus GG	484	1,246	776	160.56 ± 14.14	62.36 ± 11.10	222.92	2.57
L. casei Shirota	458	1,038	849	122.26 ± 15.66	53.95 ± 2.82	176.21	2.27

[a] e_E and e_L, number of *E. coli* and *Lactobacillus* cells adhered to 100 Caco-2 cells, respectively.

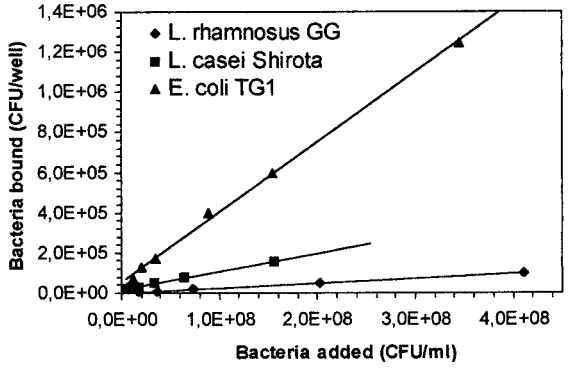

FIG. 4. Adhesion of *L. rhamnosus* GG, *L. casei* Shirota, and *E. coli* TG1 to immobilized human intestinal mucus presented as the number of bacteria bound per microtiter plate well (surface area, 0.1 cm^2) versus the concentration of bacteria added.

Displacement of adhered *E. coli* TG1 by lactobacilli. In the study, the *E. coli* TG1 (10^8 cells ml^{-1}) was allowed to adhere on Caco-2 cells. Nonadhered *E. coli* cells were removed by PBS, and then the lactobacilli (10^8 cells ml^{-1}) was added and incubated with Caco-2 cells for 1 h. The adhesion numbers of the two bacteria were counted and are shown in Table 5.

In both cases, the concentrations of the *E. coli* TG1 counted are statistically lower ($P < 0.05$) than those when the *E. coli* alone was incubated with Caco-2 cells (169.16 ± 15.63 cells/100 Caco-2 cells). However, the values of e_E/e_L were greater than 1; i.e., there were more *E. coli* cells than lactobacilli.

Adhesion of bacteria to immobilized intestinal mucus and ileostomy glycoproteins. When the number of bacterial cells adhered to intestinal mucus or human ileostomy glycoproteins per well (0.1-cm^2 wells) was plotted against the number of bacteria added per well, a near-linear to hyperbolic relationship was observed for all three of the bacteria tested (Fig. 4 and 5).

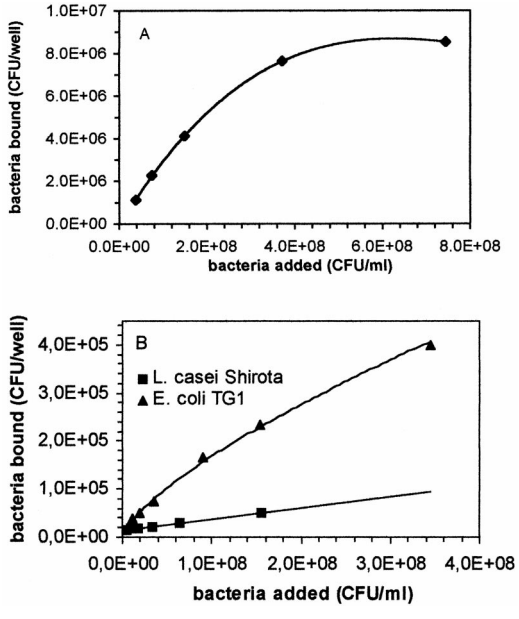

FIG. 5. Adhesion of *L. rhamnosus* GG (A) and *L. casei* Shirota and *E. coli* TG1 (B) to immobilized human ileostomy glycoproteins presented as the number of bacteria bound per microtiter plate well (surface area, 0.1 cm^2) versus the concentration of bacteria added.

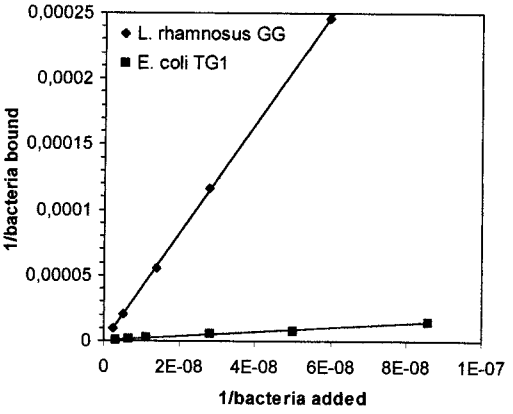

FIG. 6. Double-reciprocal representation of the adhesion of *L. rhamnosus* GG and *E. coli* TG1 to immobilized human intestinal mucus. The lines indicate the linear fit according to the least-squares method.

Double-reciprocal plots of adhered *L. rhamnosus* GG and *E. coli* TG1 against the concentration of bacteria added are presented in Fig. 6 and 7. A linear relation was observed for both plots. A double-reciprocal plot of adhered *L. casei* Shirota is shown in Fig. 8 and 9. This plot appears to be composed of two linear parts with a break at around 1.7×10^7 CFU ml^{-1} for the intestinal mucus and 9.3×10^7 CFU ml^{-1} for the human ileostomy glycoprotein. The calculated e_m and k_x values for the bacteria are shown in Table 5. For *L. casei* Shirota, the maximum number of bacterial cells that can adhere to intestinal mucus at low cell concentrations (5×10^4 cells $well^{-1}$) was four times lower than that measured at high cell concentrations (2×10^5 cells $well^{-1}$), while the maximum number of bacteria that can adhere to ileostomy glycoprotein at low cell concentrations (1.96×10^5 cells $well^{-1}$) was 5.1 times lower than that at high cell concentrations (10^6 cells $well^{-1}$). However, its adhesion affinities on intestinal mucus and ileostomy glycoproteins were higher at low cell concentrations.

DISCUSSION

Our studies indicate that the direct microscopic counting and radioactive label counting as measures of bacteria adhesion gave comparable results. The microscopic method was adopted in the studies for competition for adhesion, for it allows the differentiation of the different bacterial types on the Caco-2 cell surface. The present study demonstrated that the adhesion process of bacteria on Caco-2 cells follows the mathematical relationship (equation 2) developed for a simple dis-

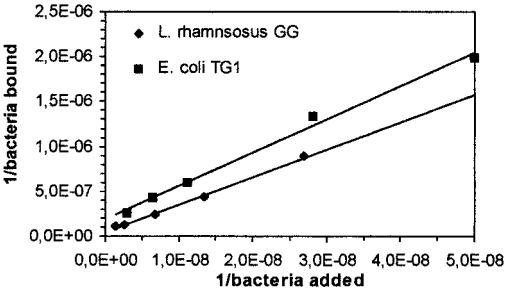

FIG. 7. Double-reciprocal representation of the adhesion of *L. rhamnosus* GG and *E. coli* TG1 to immobilized human ileostomy glycoproteins. The lines indicate the linear fit according to the least-squares method.

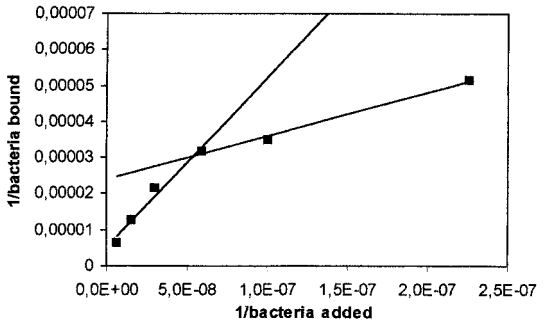

FIG. 8. Double-reciprocal representation of the adhesion of *L. casei* Shirota to immobilized human intestinal mucus. The lines indicate the linear fit according to the least-squares method.

TABLE 5. Maximum number of adhered bacterial cells to human cells and dissociation constant for various strains[a]

Specimen and strain	e_x (cells/well)	k_x (cells/well)
Intestinal mucus		
L. casei Shirota (low concn)	5×10^4	6.10×10^6
L. casei Shirota (high concn)	2×10^5	9.47×10^7
L. rhamnosus GG	2×10^7	8.28×10^{10}
E. coli TG1	1.4×10^6	2.26×10^8
Iliostomy glycoprotein		
L. casei Shirota (low concn)	1.96×10^5	2.24×10^6
L. casei Shirota (high concn)	1×10^6	1.14×10^8
L. rhamnosus GG	2×10^7	6.13×10^8
E. coli TG1	5×10^6	1.85×10^8

[a] Wells used were microtiter plate wells (0.01 cm^2). The values were calculated from equation 2 based on the data presented in Fig. 5 to 9.

sociation process involving a finite number of adhesion sites or receptors on Caco-2 cells (Fig. 2 and 3). Thus, this allows the maximum number of adhesion sites (e_m) to be estimated for each bacterial strain and the adhesion affinity of respective bacteria for Caco-2 cells to be estimated.

Among the bacteria studied, *L. rhamnosus* GG has the highest adhesion at saturation cell concentration, which is about 10 times higher than that for *L. casei* Shirota and three times higher than that for *E. coli* TG1 (Table 1). Thus, the calculations suggest that if *L. casei* Shirota and *E. coli* TG1 are competing for the same adhesion sites or surface receptors on Caco-2 cells, *L. casei* Shirota would not be able to prevent the adhesion of *E. coli*.

L. casei Shirota, on the other hand, has a higher affinity (lower dissociation constant [k_x]) than *L. rhamnosus* and *E. coli*. That is, *L. casei* Shirota adheres more readily to and dissociates less easily from Caco-2 cells.

A similar approach, applying the principles of Michaelis-Menten enzyme kinetics to the study of *E. coli* adhesion to intestinal cell monolayers, was proposed (6). It was concluded that the adhesion of *E. coli* involved an initial reversible binding step that was followed by an irreversible step. In the present study and others (4, 11, 19, 20), we had observed competition between adhered cells and free cells for adhesion sites on intestinal cells. The discrepancy between the former and the latter studies may be due to the use of undifferentiated cells in the former study.

In the studies, each of the lactobacilli was mixed with an equal number of the *E. coli* TG1 cells, and the mixed culture suspension was incubated with Caco-2 cells. If the lactobacilli and *E. coli* were competing for the same adhesion sites or surface receptors, the ratio of adhered *E. coli* (e_E) to lactobacilli (e_L) would be expected to be determined by their e_m, cell concentration added (x), and k_x as described by equation 3. This was the case when the *L. rhamnosus* GG and the *E. coli* TG1 were competing for adhesion on Caco-2 cells (Table 2). The predicted e_E/e_L values were close to the observed values obtained experimentally. The results of this study suggest that to achieve the lowest e_E/e_L, the concentration of *L. rhamnosus* should approach the saturation concentration, i.e., $>100 \times k_x$ or $>2.08 \times 10^{10}$ cells/ml. A concentration of 10^5 viable cells per ml of a probiotic product has been suggested as the therapeutic minimum for humans (16). Consumption of 10^6 to 10^{10} viable cells per day is necessary for a beneficial effect to develop (9, 16, 18). Equation 3 developed in this study suggests that the concentration of the probiotic bacterium is a critical parameter in determining e_E/e_L. It is, nevertheless, expected that a sufficient volume of a probiotic cell suspension must be consumed so that the probiotic cells can saturate the entire gastrointestinal (GI) tract, especially the lower part of the GI tract.

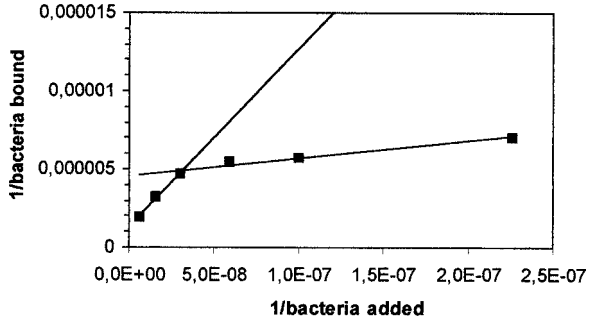

FIG. 9. Double-reciprocal representation of the adhesion of *L. casei* Shirota to immobilized human ileostomy glycoproteins. The lines indicate the linear fit according to the least-squares method.

In the study of the competition between *L. casei* Shirota and *E. coli* TG1, calculations from equation 3 predicted that the *E. coli* would compete well with *L. casei* for adhesion ($e_E/e_L > 1$), because of the lower e_m of *L. casei* (137.9 cells/100 Caco-2 cells) compared with *E. coli* (500 cells/100 Caco-2 cells). Even if *L. casei* occupied all of the 137.9 adhesion sites, it could not prevent the *E. coli* from adhering to the balance of the 362.1 sites. The observed e_E/e_L ranged between 0.135 and 0.062 (Table 3); i.e., the *Lactobacillus* prevented the adhesion of *E. coli* to Caco-2 cells. It is likely that the interaction of *L. casei* and *E. coli* involved more than competition for the adhesion sites on Caco-2 cells.

In the exclusion study, it was observed that *L. rhamnosus* GG did not prevent the adhesion of *E. coli* to Caco-2 cells. This may be due to the relatively high dissociation constant of *L. rhamnosus* (2.08×10^8 cells ml^{-1}), and any dissociated *Lactobacillus* cells were readily replaced by surrounding *E. coli* cells in the culture medium. The concentration of *E. coli* determined (122.26 ± 15.66 cells/100 Caco-2 cells) in the study involving *L. casei* was slightly, though statistically significantly ($P < 0.05$) lower than the concentration determined when *E. coli* was present alone (169.16 ± 15.63 cells/100 Caco-2 cells). The lower dissociation constant (8.62×10^7 cells ml^{-1}) and possibly a soluble protein factor produced by *L. casei* Shirota may have hindered their displacement by *E. coli* cells. This observation is in agreement with studies of humans and animals, where *Lactobacillus* cells were found to be replaced gradually by enterobacteria after the intake of lactobacilli had

TABLE 6. Results of the displacement study in which *E. coli* TG1 cells were allowed to adhere to Caco-2 cells before lactobacilli were added[a]

Strain	Total no. of cells counted			Observed $e_E \pm$ SD	Observed $e_L \pm$ SD	Observed $e_E + e_L$	Observed e_E/e_L
	Adhered *Lactobacillus*	Adhered *E. coli*	Caco-2				
L. rhamnosus GG	209	1,183	982	120.47 ± 15.56	98.30 ± 11.30	141.75	5.66
L. casei Shirota	158	984	1,001	21.28 ± 4.10	15.78 ± 7.07	114.08	6.23

[a] See footnote *a* to Table 4 for explanation of e_E and e_L.

stopped (11, 19). Commercially available probiotic organisms have not been reported to establish themselves permanently in the human GI tract. Our results suggest that the concentration of free (nonadhering) *Lactobacillus* in the GI contents needs to be maintained at a high level to prevent the adhered lactobacilli from being replaced by other bacteria; alternatively, the lactobacilli need to divide rapidly to maintain a high local cell concentration. The observation that adhered *Lactobacillus* cells in the GI tract were gradually replaced by enterobacteria suggests that the lactobacilli used were not able to grow sufficiently rapidly to establish permanent residence in the GI tract.

There is ample laboratory and clinical evidence to demonstrate that oral administration of lactobacilli could be used to treat GI bacterial infection (9). The efficacy of lactobacilli in displacing adhered *E. coli* on Caco-2 cells was investigated in the displacement study (Table 6). The process of displacing adhered *E. coli* with *Lactobacillus* appears to be slow. After 1 h of incubation with lactobacilli alone, less than half of the adhered *E. coli* cells were displaced. In the studies using fermented milk containing lactobacilli to treat diarrheal disorders in human patients (4, 12, 15), it was necessary that patients consume the fermented milk for 2 to 3 days, before significant improvement in clinical symptoms of the illness was observed. It is important to recognize the slow process of displacing *E. coli* with lactobacilli in the in vitro study, which could not simply be explained by dissociation phenomena.

Although *L. rhamnosus* GG had the highest saturation cell concentration, its adhesion affinity to intestinal mucus and ileostomy glycoproteins was considerably lower than that of *L. casei* Shirota or *E. coli* TG1. The double-reciprocal plots of *L. casei* Shirota added versus bound, allowed two linear curve fits (Fig. 8 and 9). This may mean that two binding mechanisms are involved for this bacterium, one for a high bacterial concentration (lower affinity) and one for a lower concentration (higher affinity). A possible explanation is that multiple adhesion sites on the mucous layer are involved in the adhesion of an *L. casei* Shirota cell. At low bacterial concentrations, a bacterium adheres to a maximum number of sites on mucus, whereas at high bacterial concentrations, a minimum number of adhesion sites are involved. The data in Table 5 suggest that up to four adhesion sites ($2 \times 10^5/5 \times 10^4 = 4$) on intestinal mucus and five adhesion sites on ileostomy glycoproteins could be involved in the adhesion of *L. casei* Shirota on intestinal mucus. This explains the observation that at low bacterial concentrations the affinity for mucus was high, whereas at high bacterial concentrations the adhesion affinity was lower (Table 5). In either case, the adhesion affinity is much higher than those for the other two tested bacteria.

The quantitative approach developed in this study has proven useful in the understanding of the mechanism and kinetics of the adhesion process of bacteria on intestinal cells and mucus and the competition between different bacteria for adhesion. Our results may provide help in estimating the numbers of bacteria needed for future competitive exclusion studies.

ACKNOWLEDGMENTS

The skillful technical assistance of Satu Tölkkö is gratefully acknowledged.

REFERENCES

1. **Benno, Y., and T. Mitsuoka.** 1992. Impact of *Bifidobacterium longum* on human fecal microflora. Microbiol. Immun. **36:**683–694.
2. **Bernet, M.-F., D. Brassart, J.-R. Neeser, and A. L. Servin.** 1993. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. Appl. Environ. Microbiol. **59:**4121–4128.
3. **Bernet, M.-F., D. Brassart, J.-R. Neeser, and A. L. Servin.** 1994. *Lactobacillus acidophilus* LA1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. Gut **35:**483–489.
4. **Boudraa, G., M. Touhami, P. Pochart, R. Soltana, J. Y. Mary, and J. F. Desjeux.** 1990. Effect of feeding yogurt versus milk in children with persistent diarrhea. J. Pediatr. Gastroenterol. Nutr. **11:**509–512.
5. **Chauviere, G., M.-H. Coconnier, S. Kerneis, J. Fourniat, and A. L. Servin.** 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J. Gen. Microbiol. **138:**1689–1696.
6. **Cohen, P. S., A. D. Elbein, R. Solf, H. Mett, J. Regos, E. B. Menge, and K. Vosbeck.** 1981. Michaelis-Menten kinetic analysis of *Escherichia coli* SS142 adhesion to intestine 407 monolayers. FEMS Microbiol. Lett. **12:**99–103.
7. **Fogh, J., J. M. Fogh, and T. Orfeo.** 1977. One hundred and twenty-seven cultured human tumour cell lines producing tumours in nude mice. J. Natl. Cancer Inst. **59:**221–226.
8. **Greene, J. D., and T. R. Klaenhammer.** 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl. Environm. Microbiol. **60:**4487–4494.
9. **Lee, Y. K., K. Nomoto, S. Salminen, and S. L. Gorbach.** 1999. Handbook of Probiotics. John Wiley & Sons, New York, N.Y.
10. **Lee, Y. K., and S. Salminen.** 1995. The coming of age of probiotics. Trends Food Sci. Technol. **6:**241–244.
11. **Lidbeck, A., J.-A. Gustafasson, and C. Nord.** 1987. Impact of *Lactobacillus acidophilus* supplements on the human oropharyngeal and intestinal microflora. Scand. J. Infect. Dis. **19:**531–537.
12. **Majamaa, H., E. Isolauri, M. Saxelin, and T. Vesikari.** 1995. Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. J. Pediatr. Gastroenterol. Nutr. **20:**333–338.
13. **Ouwehand, A. C., P. Niemi, and S. J. Salminen.** 1999. The normal fecal microflora does not affect the adhesion of probiotic bacteria in vitro. FEMS Microbiol. Lett. **177:**35–38.
14. **Pinto, M., S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum.** 1983. Enterocyte-like differentiation and polarisation of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell **47:**323–330.
15. **Raza, S., S. M. Graham, S. J. Allen, S. Sultana, L. Cuevas, and C. A. Hart.** 1995. *Lactobacillus* GG promotes recovery from acute nonbloody diarrhea in Pakistan. Pediatr. Infect. Dis. J. **14:**107–111.
16. **Robinson, R. K. (ed.).** 1991. Therapeutic properties of fermented milks, p. 23–43. Elsevier Applied Science, London, United Kingdom.
17. **Salminen, S., A. Ouwehand, Y. Benno, and Y. K. Lee.** 1999. Probiotics: how should they be defined? Food Sci. Technol. **10:**107–110.
18. **Salminen, S., M. Deighton, and S. Gorbach.** 1993. Lactic acid bacteria in health and disease, p. 199–225. *In* S. Salminen and A. von Wright (ed.), Lactic acid bacteria. Marcel Dekker, New York, N.Y.
19. **Saxelin, M., T. Pessi, and S. Salminen.** 1995. Fecal recovery following oral administration of *Lactobacillus* strain GG (ATCC 53103) in gelatine capsules to healthy volunteerts. Int. J. Food Microbiol. **25:**199–203.
20. **Tamura, N., M. Norimoto, K. Yoshida, C. Hirayama, and R. Nakai.** 1983. Alteration of fecal bacterial flora following oral administration of bifidobacterial preparation. Gastroenterol. Jpn. **18:**17–24.
21. **Tuomola, E. M., and S. J. Salminen.** 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int. J. Food Microbiol. **41:**45–51.