Lectin Activity and Chemical Characteristics of *Escherichia coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* from Gastrointestinal Mucosa of Growing Pigs*

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ABSTRACT : Lectin activities and chemical characteristics of *Escherichia coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* originating from the porcine cecal mucosal layer were studied based on hemagglutination assay (HA) and hemagglutination inhibition assay (HIA). Although all the bacterial strains were able to agglutinate erythrocytes of porcine or rabbit origin, much higher HA titers were consistently observed for *Lactobacillus spp.* than for *E. coli* or for *Bifidobacterium spp.* A remarkable reduction in HA titers occurred by the treatment of *E. coli* and *Lactobacillus spp.* with protease or trypsin and of *Bifidobacterium spp.* with protease, trypsin or periodate. There were no significant effects on the HA titers of the three groups of bacteria after the treatment with lipase. Hemagglutination of *E. coli* was strongly inhibited by D (+)-mannose and D (+)-galactose; *Lactobacillus spp.* by α-L-rhamnose and methyl-β-galactopyranoside; *Bifidobacterium spp.* by D (+)-galactose, α-L-rhamnose, α-L-fucose, L (+)-arabinose, D (+)-mannose, D (-)-fructose at a relatively low concentration (1.43 to 3.75 mg/ml). These results, combined with the enhanced HA activities of the three bacterial strains by modification of rabbit erythrocytes with neuraminidase and abolished HA activity of *E. coli* after treatment with β-galactosidase, indicate that it might be the glycoproteinous substances surrounding the surface of the bacterial cells that are responsible for the adhesions of these microorganisms by recognizing the specific receptors on the red blood cell. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 6 : 863-868)***

Key Words : Indigenous Gastrointestinal Bacteria, *Escherichia coli*, *Lactobacillus spp.*, *Bifidobacterium spp.*, Lectins, Growing Pigs

INTRODUCTION

The commensal biota of the pig gut has been widely studied, in part because it is thought to have a role in protecting pigs against disease. The principal components of the fecal biota of pigs are *streptococci*, *lactobacilli*, *eubacteria*, *fusobacteria*, *bacteroides*, *peptostreptococci*, *bifidobacteria*, *selenomonas*, *clostridia*, *butyrivibrio* and *escherichia* (Stewart, 1996). The functions of competitive exclusion of inherent microorganisms in the gut against pathogens have been established and some species of these bacteria such as *lactobacilli* or *bifidobacteria* have been included in direct-fed microbes (DFM) or probiotics (Hentges, 1992), and they are used to balance the intestinal flora and to prevent several gastrointestinal disorders (Yoon and Won, 2002). But information about the mechanisms of bacterial colonization is limited. A stable attachment of the bacteria to the intestinal epithelium of pigs before invasion seems essential for initiation of the infection. Bacterial attachment to living cells is mediated by lectins located on bacterial surfaces (Sharon and Lis, 1972; Sharon, 1987). Lectins have been defined as sugar-binding proteins or glycoproteins of non-immune origin that agglutinate cells or precipitate saccharides or glycoconjugates (Goldstein et al., 1980).

It has been established that lectins exist on the surface of rumen bacteria, and that these lectins have carbohydrate- and glycoprotein-binding specificity (Meng and Preston, 1997). Also lectins may mediate the attachment of rumen bacteria to rumen epithelia (Meng and Preston, 1998). Another experiment using *Bifidobacterium pseudolongum*, enteric *Escherichia coli K88* or *Salmonella choleraesuis* of piglet origin also demonstrated that proteinaceous or glycoproteinaceous lectin-like substances that recognize galactosyl residue-containing molecules, especially intestinal mucin, exist on the surface of such bacteria (Meng et al., 1998). So we postulated that the indigenous bacteria of lactobacilli or bifidobacteria in the mucosal layers might be directly relative to high lectin activities. The purpose of this study was to determine the differences in lectin or lectin-like activities and characteristics among these microorganisms.

METERIALS AND METHODS

Bacteria and cultural condition

Escherichia coli, *Lactobacillus spp.* and *Bifidobacterium spp.* used in our experiments were isolated from the mucosal layer of the cecum of growing pigs previously in this laboratory and tentatively identified. Primary culture of *E. coli*, *Lactobacillus spp.* and

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Bifidobacterium spp. were grown overnight in nutrient broth, modified Rogosa SL (MRS) broth and reinforced clostridium agar (RCA) (Alm et al., 1989), respectively at 37°C. The anaerobic incubation was performed in an anaerobic chamber (N_2:CO_2:H_2=75:15: 10; Model 1025, Forma Scientific Products, Marietta, OH, USA). MacConkey agar (Difco) plates for *E. coli*, MRS agar plates for *Lactobacillus spp.* and RCA agar plates for *Bifidobacterium spp.* were prepared anaerobically by the method of Hungate (1969). Pure cultures were harvested from ten agar plates for each bacterial strain after culturing for 48 h by scraping cells from the plates. The harvested cells were then centrifuged at 20,000×g for 20 min and washed twice with phosphate buffered saline (PBS, pH 7.2, 0.01 M). After washing, all bacterial pellets were re-suspended in PBS and cell density was adjusted to 10^{10} cells per ml with a regular counting chamber method.

Erythrocyte preparation

Fresh blood was taken from a healthy piglet and a rabbit into Alsever's solution (Tocuda and Warringtong, 1970). The cells were washed five times with PBS (pH 7.2) by centrifugation at 740×g for 10 min at 4°C. The buffy coat was carefully removed. The washed erythrocytes were re-suspended in PBS to a final concentration of 2% and stored at 4°C until use.

Hemagglutination assays

The hemagglutination (HA) assays were carried out at room temperature using PBS as a diluent as described by Mukai et al. (1992). All assays were performed in triplicate. Bacterial preparations (25 µl) were serially diluted twofold with PBS in a U-bottom style microtiter plate with 96 wells (Dingguo Biotechnology, Beijing, China), followed by incubation with an equal volume of 2% suspension of erythrocytes in PBS at room temperature for 2 h. Hemagglutination titers were taken as the maximum dilution at which erythrocytes were completely agglutinated.

Hemagglutination inhibition assays

The hemagglutination inhibition assays were performed according to the method described by Meng and Preston (1997). Sixteen saccharides (D (+)-glucose, D (+)-galactose, α-L-rhamnose, α-L-fucose, L (+)-arabinose, D (+)-xylose, D (+)-mannose, D (-)-fructose, D-lactose, maltose, sucrose, D (+)-cellobiose, N-acetyl-D-galactosamine, methyl-α-galactopyranoside, methyl-β-galactopyranoside and fructooligosaccharide; all from Sigma Chemical Company, St. Louis, MO) and three glycoproteins (Bovine submaxillary mucin, Sigma type I-S; thyroglobulin from porcine, Sigma; porcine stomach mucin, Sigma type III) were used as potential inhibitors of agglutination by the bacterial strains. All reagents were dissolved in PBS to a concentration of 150 mg/ml for saccharides, except the concentrations of lactose, cellobiose and methyl-β-galactopyranoside which were 120, 75 and 40 mg/ml, respectively. The concentration of all glycoproteins was 12 mg/ml. Serially diluted inhibitors (25 µl) in PBS were mixed with an equal volume of bacterial suspension on the microtiter plates and incubated for 30 min at 37°C. Afterwards, 25 µl of a 2% erythrocyte suspension were added to the mixture in each well. All plates were vortexed and left standing at room temperature for 2 h. The substances serially diluted in the well where agglutinations were completely inhibited were determined as agglutination inhibitors.

Enzymatic and chemical treatment of bacteria

Two milliliters of bacterial suspensions of each bacterium were suspended in 2 ml of trypsin (2.5 mg/ml; Sigma), protease (0.4%; Sigma type XXIII, from *Aspergillus oryzae*), lipase (0.4%; Sigma), periodate (20 mM sodium metaperiodate) or ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA, 0.04%) in PBS, respectively. The same amount (2 ml) of bacterial suspensions suspended in 2 ml of PBS at the same time as untreated controls. The suspensions were incubated in a 39°C warm water bath for 60 min. After incubation finished, the suspensions were centrifuged at 20,000×g for 20 min at 4°C and bacterial pellets were washed five times with PBS. The final bacterial cell density was adjusted with PBS in the same way as before and HA assay was conducted.

Modification of erythrocytes

Enzymatic modification of rabbit erythrocytes was carried out with neuraminidase (Sigma Type V, from *Clostridium perfringens*), β-galactosidase (Roche, from *E. coli* overproducer) or 1% of glutaraldehyde solution (25% aqueous solution). One milliliter of 10% washed rabbit erythrocyte suspension was mixed with 2 ml of enzyme solution (1 mg of enzyme per ml of PBS) of neuraminidase or β-galactosidase and incubated at 37°C for 60 min. The modification with glutaraldehyde was carried out using the method of Meng and Preston (1997). One milliliter of 10% rabbit erythrocyte suspension was mixed with an equal volume of 1% glutaraldehyde solution in PBS which was freshly prepared and cooled to 4°C. The suspension was magnetically stirred at 4°C for 1 h. All erythrocytes were then washed five times in PBS by centrifugation at 740×g for 10 min at 4°C and re-suspended with PBS to a final 2% suspension.

Statistical analysis

The experiments for agglutination of pig and rabbit erythrocytes by the bacterial strains and the treatment of

Table 1. Effect of chemical and enzymatic treatment of *Escherichia coli*, *Lactobacillus spp.* or *Bifidobacterium spp.* on their hemagglutination titers*

Treatment	Bacteria		
	Escherichia coli	*Lactobacillus spp.*	*Bifidobacterium spp.*
Protease	2.67±1.00^{c}	6±2.09^{c}	2.0±0.00^{c}
Trypsin	2.00±0.00^{c}	5±1.81^{c}	3.0±1.1^{c}
Lipase	57.6±102.8^{b}	36.17±55.51^{b}	6.4±1.96^{b}
Periodate	5.5±2.71^{c}	3.5±0.90^{c}	NHA^{c}
EDTA	8.67±6.08^{b}	4.53±2.67^{c}	4.0±0.00^{c}
Control	6.8±2.48^{b}	12.4±10.01^{b}	5.2±2.48^{b}

* Results were expressed as the mean of hemagglutination titers±standard error (n=3); NHA-no visible hemagglutination; Means in a column without common letters significantly differ ($p<0.01$).

Table 2. Effect of modification of rabbit erythrocytes on HA titers of *Escherichia coli*, *Lactobacillus spp.* and *Bifidobacterium spp.**

Bacteria species	Modification of erythrocytes by			
	Control	Glutaraldehyde	β-Galactosidase	Neuraminidase
Escherichia coli	3.6±0.83^{c}	0^{d}	0^{d}	16.4±9.83^{b}
Lactobacillus spp.	13.07±12.5^{c}	0^{d}	8.8±6.09^{c}	60.8±56.8^{b}
Bifidobacterium spp.	4.0±0.0^{c}	4.8±1.66^{c}	4.4±2.03^{c}	15.47±9.30^{b}

* Results were expressed as the mean of hemagglutination titers±standard error (n=3); Means within a row without common letters significantly differ ($p<0.01$).

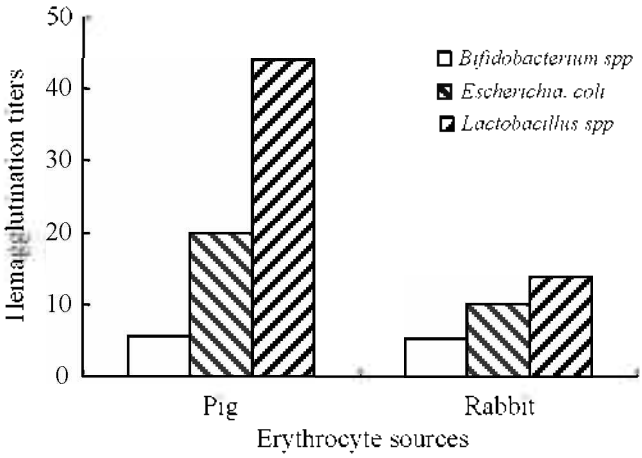

Figure 1. Hemagglutination of animal erythrocytes by *Escherichia coli*, *Lactobacillus spp* and *Bifidobacterium spp.* Results shown in the figure are expressed as the means of hemagglutination titers of *Escherichia coli*, *Lactobacillus spp* and *Bifidobacterium spp* from pig origin (n=3). The HA titer was expressed as the greatest dilution of bacterial suspension at which erythrocytes were still completely agglutinated.

bacteria and the modification of erythrocytes for hemagglutination were all performed three times with each time in triplicate. Data from all experiments were analyzed by the GLM procedure of SAS for a factorial design (SAS, 1996). The model of HA titers included bacterial species, erythrocyte sources, treatment of bacteria, modification of erythrocytes, and the possible interactions between them in an individual experiment. Since the sugars and glycoproteins used could not be taken as a treatment factor, the data were not included in the statistical analysis for the HA inhibition experiments.

RESULTS

The results of agglutination of erythrocytes by *E. coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* are shown in Figure 1. Bacterial species, erythrocyte sources, and their interactions significantly ($p=0.001$) influenced HA activities of the bacteria. Although all bacterial species agglutinated pig and rabbit erythrocytes, *Lactobacillus spp.* showed a stronger ($p<0.001$) agglutinating activity than *E. coli* and *Bifidobacterium spp.* from pig origin. For both sources of erythrocytes investigated, there were significant differences of agglutinating rabbit erythrocytes by the three species of bacteria ($p<0.01$), but no difference in agglutinating pig erythrocytes was observed between *E. coli* and *Bifidobacterium spp.* Consequently, the rabbit erythrocytes were adopted as a model for the following experiments in our studies.

Chemical and enzymatic treatments of bacterial cells significantly influenced HA titers of the three groups of bacteria (Table 1). The HA titers of *E. coli* were significantly decreased by the treatments with protease ($p<0.001$), trypsin ($p<0.001$), and periodate ($p=0.033$); the treatment with EDTA ($p=0.372$) or lipase ($p=0.662$) had no effect on the HA titers. The HA activities of *Lactobacillus spp.* were significantly decreased after the treatments with protease ($p=0.009$), trypsin ($p=0.004$), periodate ($p=0.001$), and EDTA ($p=0.006$), but were not affected by lipase ($p=0.232$). The HA titers of *Bifidobacterium spp.* also were significantly diminished or totally abolished by the treatment with protease ($p<0.001$), trypsin ($p<0.001$), periodate ($p<0.001$) and EDTA ($p=0.001$) except for lipase ($p=0.158$) compared with the untreated control.

Modification of rabbit erythrocytes with neuraminidase considerably enhanced agglutination of the erythrocytes by *E. coli* ($p<0.001$), *Lactobacillus spp.* ($p=0.004$) and *Bifidobacterium spp.* ($p<0.001$) relative to the control (Table 2). When the erythrocytes were modified with glutaraldehyde, HA activity was completely destroyed for *E. coli* ($p<0.001$) and *Lactobacillus spp.* ($p<0.001$) but it did not significantly change for *Bifidobacterium spp.* ($p=0.072$). The modification of rabbit erythrocytes with *β-galactosidase* totally decreased the HA titers for *E. coli* strains ($p<0.001$), but there were no effects on *Lactobacillus spp.* ($p=0.245$) and *Bifidobacterium spp.* ($p=0.451$).

Among the sugars tested in the HA inhibitory experiments (Table 3), D (+)-mannose and D (+)-galactose were found to be inhibitors for the HA of *E. coli*; α-L-rhamnose and methyl-β-galactopyranoside were discovered to be inhibitors for *Lactobacillus spp.*; whereas D (+)-galactose, α-L-rhamnose, α-L-fucose, L (+)-arabinose, D (+)-mannose, D (-)-fructose were revealed to be the inhibitors for *Bifidobacterium spp.*. Glycoproteins, including bovine submaxillary, thyroglobulin and porcine stomach mucin did not exhibit the inhibitory effect on the HA of the three microorganisms.

DISCUSSION

E. coli, *Lactobacillus spp.* and *Bifidobacterium spp.* agglutinated pig and rabbit erythrocytes (Figure 1) and this hemagglutination can be specifically inhibited by some sugars. Thus, this study clearly demonstrated that lectin-like components exist on the surface of *E. coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* of porcine origin based on the definition of lectins (Goldstein et al., 1980). Although the exact function of bacterial lectins is not clear, it is thought that they play a key role in mediating attachment of bacteria to their host cells (Sharon, 1987). In the present study, a higher HA titer was detected in strains of *Lactobacillus spp.* than in strains of *E. coli* or *Bifidobacterium spp.*, independent of the sources (pig and rabbit) of erythrocytes used, suggesting that *Lactobacilli* may have a preference of attachment to the pig intestinal epithelium over *E. coli* or *Bifidobacteria*. Such preference would allow lactobacilli to firmly attach to the mucosa of intestinal epithelium of host animal and thereby block colonization by any pathogenic organisms.

Glycoproteinaceous components located on the bacterial surface of *E. coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* might be responsible for the lectin-like activities, because the HA titers were reduced significantly after the chemical or enzymatic treatments (except for lipase) of these bacterial strains. These results implied that certain glycoproteinaceous components, rather than proteins or glycolipids, might be responsible for the HA of the three groups of microorganisms. Wadstrom et al. (1987) observed that *Lactobacillus* strain *aa* treated with protease or heated prior to protease treatment showed a remarkably reduction not only in the ability to adhere to the intestinal epithelial cells of pigs but also in the surface hydrophobicity (Wadstrom et al., 1987). Henriksson et al. (1991) reported that treatment of *Lactobacillus fermentum* strain 104-S with

Table 3. Inhibitory activities of some saccharides and glycoproteins on agglutinations of rabbit erythrocytes by *Escherichia coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* from pig origin*

Inhibitors	Minimum amount of inhibitor required for inhibition, mg/ml		
	Escherichia coli	*Lactobacillus spp.*	*Bifidobacterium spp.*
D (+)-Glucose	>25	>25	>25
D (+)-Galactose	2.53±0.99	>25	2.3±1.01
α-L-Rhamnose	6.02±2.49	1.45±1.08	2.2±0.81
α-L-Fucose	12.29±10.80	18.75±6.85	3.14±1.78
L (+)-Arabinose	3.64±1.43	>25	3.44±1.60
D (+)-Xylose	>25	>25	>25
D (+)-Mannose	1.43±0.34	>25	3.74±1.32
D (-)-Fructose	6.28±2.71	5.53±4.00	3.98±2.08
D-Lactose	5.83±3.62	>20	5.00±0.00
Maltose	15.01±6.59	14.39±6.59	>25
Sucrose	14.81±9.02	18.75±6.85	7.54±2.57
D (+)-Cellobiose	>12.5	>12.5	>12.5
N-Acetyl-D-Galactosamine	>0.83	>0.83	>0.83
Methyl-α-galactopyranoside	9.79±8.68	4.95±4.60	5.23±1.56
Methyl-β-galactopyranoside	4.66±1.72	3.75±1.91	5.79±1.56
FOS	>25	11.14±8.46	>25
Bovine submaxillary mucin	>2	>2	>2
Thyroglobulin	>2	>2	>2
	>2	>2	>2

* Results were expressed as the mean of hemagglutination titers±standard error (n=3).

metaperiodate did not affect the adhesion to porcine gastric squamous epithelium or polystyrene; however, protease treatment dramatically decreased the adhesion of both strains 104-S and 104-R, suggesting that the determinants responsible for the adhesion were proteinaceous. Carbohydrates may be partially involved in the adhesion of 104-R, because metaperiodate-treated cells adhered more poorly than control, iodate-treated cells (Henriksson et al., 1991). However, in the present experiment, we found that metaperiodate treatment exhibited a remarkable reduction of hemagglutination titers. *Bifidobacterium spp.* strains showed a significant reduction in comparison to *E. coli* and *Lactobacillus spp.*, suggesting that glycoproteinaceous components may be responsible for the hemagglutination ability of these bacteria strains and that also more carbohydrates may be involved in the agglutination of the bifidobacterium. The lectin activity of *E. coli* strains after the treatment with EDTA solution decreased significantly, implying that certain divalent cation bridging is probably required for the HA reaction by this bacterium. This result is consistent with a previous study of Meng and Preston (1998). Lipase-treatment could not enhance the lectin activities of bacterial strains in the present study, which is not in agreement with the previous study conducted by Meng et al. (1998). These contradictory results may be attributed to these bacterial strains used in these experiments.

In an attempt to characterize lectin receptors of the three bacterial species, we subjected rabbit erythrocytes to enzymatic modification. Neuraminidase treatment can expose galactose residues on the erythrocyte surface. Modification with neuraminidase caused a remarkable increase in HA activity of *E. coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* strains. This result is in accordance with a previous study of Meng et al. (1998). Wadstrom et al. (1987) also revealed that neuraminidase-treated epithelial cells of pigs bound more lactobacilli compared with untreated cells. This result suggests that galactose residues present on erythrocytes are a specifically binding substance for lectins of *E. coli*, *Lactobacilli* and *Bifidobacteria*. β-Galactosidase modification of erythrocytes completely abolished the HA activity of *E. coli*, but did not affect that of *Lactobacillus spp.* and *Bifidobacterium spp.*. Meng and Preston (1997) also found that modification of cattle erythrocytes with β-galactosidase has no changes in the titers of rumen bacteria before or after modification. A possible reason is that these strains can express lectins with different sugar specificities and neuraminidase treatment may uncover residues other than galactose. When rabbit erythrocytes were modified with glutaraldehyde, the HA activities of *E. coli* and *Lactobacillus spp.* were totally abolished; however, no effect was determined on the HA activity of *Bifidobacterium spp.* compared with the control, implying that cross-linking and stabilizing the protein part of erythrocyte membranes would influence the recognition of *E. coli* and *Lactobacilli* lectins with their specific receptors. These results indicated that galactosyl residue-containing glycoproteins on the surface of rabbit erythrocytes might serve as receptors for the bacterial lectin-like components.

Agglutination reactions were investigated for inhibition by saccharides and glycoproteins (Table 3). All sixteen saccharides and three kinds of glycoproteins were included in the hemagglutination inhibition assays. The hemagglutination reaction of *E. coli* could be inhibited by mannose (1.43 mg/ml) and galactose (2.53 mg/ml) specifically; the hemagglutination reaction of *Lactobacillus spp.* could be inhibited by rhamnose (1.45 mg/ml) and methyl-β-D-galactopyranoside (3.75 mg/ml) specifically; hemagglutination reaction of *Bifidobacterium spp.* could be inhibited by rhamnose (2.2 mg/ml), galactose (2.3 mg/ml), fucose (3.14 mg/ml), arabinose (3.44 mg/ml), mannose (3.74 mg/ml) and fructose (3.98 mg/ml), respectively. *Bifidobacterium spp.* has more specific inhibitors than the other two bacteria, indicating that there are various mono- or di-saccharides located on the surface of rabbit erythrocytes as receptors on bacterial lectins. Based on these observations, we hypothesize that bifidobacterium may have more adhesion sites on the epithelium cells of intestinal mucosa of pigs. All of the glycoproteins employed in our experiment have no inhibitory effects on the hemagglutination reaction. This result is inconsistent with previous studies of Meng and Preston (1997) and Meng et al. (1998). Glycoproteins are the major components of porcine stomach mucin, bovine submaxillary mucin and thyroglobulin (Meng et al., 1998), implying that sugars, not glycoproteins are the specific receptors of bacterial lectins.

Finally, the results of this study demonstrate that lectins exist on the surface of *E. coli*, *Lactobacillus spp.* and *Bifidobacterium spp.* and that these lectins have carbohydrate-binding specificity. Due to a specific recognition and combination with complementary saccharide residues of glycoproteins on the membrane of host cells and the relatively higher lectin activities of *Lactobacillus spp.* and relatively more adhesion sites of *Bifidobacterium spp.*, we postulate that this function may play a key role for their colonization in the intestinal mucus of host animals. An important role of the indigenous intestinal microflora is to exert a barrier against attachment and colonization of the gut epithelium by pathogenic bacteria (Chapman, 1988; Hentges, 1992). Lactobacilli and bifidobacteria are two major species of the intestinal microflora (Savage, 1977; Simon and Gorbach, 1986). Furthermore, *Bifidobacteria* of human intestinal origin were shown to attach to cultured human intestinal epithelial cells and inhibit the attachment by enteropathogenic bacteria

(Bernet et al., 1993). One of the mechanisms for indigenous intestinal microorganisms to prevent attachment and colonization of the intestinal epithelium by enteropathogens has been proposed to be related to saturation of the adhesion receptors in the epithelium by indigenous microflora and thus prevent attachment by pathogenic organisms (Stavric and Kornegay, 1995). Therefore, understanding of the adhesion mechanisms of indigenous bacteria may help us to maintain a predominant community of beneficial microflora along the intestinal mucosa and to prevent the colonization with pathogenic bacteria.

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