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Assessment of Lipopolysaccharide-binding Activity of *Bifidobacterium* and Its Relationship with Cell Surface Hydrophobicity, Autoaggregation, and Inhibition of Interleukin-8 Production

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Abstract This study was performed to screen probiotic bifidobacteria for their ability to bind and neutralize lipopolysaccharides (LPS) from *Escherichia coli* and to verify the relationship between LPS-binding ability, cell surface hydrophobicity (CSH), and inhibition of LPS-induced interleukin-8 (IL-8) secretion by HT-29 cells of the various bifidobacterial strains. Ninety bifidobacteria isolates from human feces were assessed for their ability to bind fluorescein isothiocyanate (FITC)-labeled LPS from *E. coli*. Isolates showing 30–60% binding were designated LPS-high binding (LPS-H) and those with less than 15% binding were designated LPS-low binding (LPS-L). The CSH, autoaggregation (AA), and inhibition of LPS-induced IL-8 release from HT-29 cells of the LPS-H and LPS-L groups were evaluated. Five bifidobacteria strains showed high levels of LPS binding, CSH, AA, and inhibition of IL-8 release. However, statistically significant correlations between LPS binding, CSH, AA, and reduction of IL-8 release were not found. Although we could isolate bifidobacteria with high LPS-binding ability, CSH, AA, and inhibition of IL-8 release, each characteristic should be considered as strain dependent. Bifidobacteria with high LPS binding and inhibition of IL-8 release may be good agents for preventing inflammation by neutralizing Gram-negative endotoxins and improving intestinal health.

Keywords: Aggregation, bifidobacteria, cell surface hydrophobicity, interleukin-8, lipopolysaccharides

Probiotic bifidobacteria exert health-promoting effects on the host by maintaining intestinal microflora balances, reducing lactose intolerance and serum cholesterol levels, increasing vitamin synthesis, and boosting the immune system of the host [17]. Adherence to epithelial cells and mucosal surfaces is an important property of bifidobacteria as probiotics, because adhesion to the intestinal epithelium is an important prerequisite for colonization of the gastrointestinal tract by probiotic strains, and prevents their immediate elimination by peristalsis, thus providing a competitive advantage in this ecosystem [1, 10, 15, 23]. Cell surface hydrophobicity (CSH) and autoaggregation (AA) of probiotic strains appear to be involved in their interactions with their surrounding environments, and coaggregation abilities may constitute a barrier that prevents colonization by pathogenic microorganisms [3, 8].

Del Re *et al.* [8] reported that autoaggregation of bifidobacteria was related to their surface hydrophobicity. On the other hand, when Enrica *et al.* [9] investigated the relationship between CSH, AA, and the contact angle of 30 bifidobacteria strains, no relation between CSH and contact angle was observed. These contradictory results suggest that more studies are needed to better understand the surface properties of bifidobacteria.

Probiotic formulas containing bifidobacteria have been shown to be effective in reducing the severity of inflammation in several animal models and patients with inflammatory bowel disease (IBD) [2, 11, 12, 19, 20].

Inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC) accompany disruption in intestinal microflora [13] and mucosal inflammation [8]. One of the important mediating factors for IBD is nuclear factor κB (NF-κB), which is stimulated by tumor necrosis factor α (TNF-α), IL-1β, or LPS. LPS is conveyed by LPS-binding protein (LBP) and the intermediate receptor CD14, which then promotes binding of LPS to toll-like receptor 4 (TLR4) and MD-2. It has been reported that expression of TLR4 and CD14 is significantly increased in

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IECs of animal models of colitis [21, 22] and IBD patients [4].

The aim of this study was to screen for bifidobacteria with high LPS-binding abilities and characterize the relationships between CSH, AA, and inhibition of interleukin-8 secretion. Ninety bifidobacteria isolates from human feces were screened to determine if those with more hydrophobic surfaces were better able to bind *E. coli* LPS and inhibit LPS-induced cytokine IL-8 release from human HT-29 cells.

MATERIALS AND METHODS

Reagents and Bacterial Culture Conditions

LPS and FITC-labeled LPS from *E. coli* serotype O111:B4 were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). A human IL-8 ELISA kit (OptEIA) was purchased from Pharmingen (San Diego, CA, U.S.A.). Ninety bifidobacteria isolates from human feces were provided by the Research Center at BIFIDO Co., Ltd. (KangWon, Korea) and were named NO.01-90. Bifidobacteria were anaerobically cultured on MRS agar plates for single colony isolation and then activated twice in MRS broth [6] at 37°C for 18 h before being harvested by centrifugation at 8,000 ×*g* for 5 min. The bacterial pellets were washed twice in phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS or culture medium.

Identification of Bifidobacteria at the Genus Level Using 16S rRNA Gene

The 90 strains used in this study were confirmed to be bifidobacteria using the fructose 6-phosphate phosphoketolase (F6PPK) test carried out according to the procedure of Scardovi [26]. The genomic DNA from each bifidobacteria was isolated using the Instagene DNA purification matrix (Bio-Rad), according to the manufacturer's instructions. The isolated strains were also confirmed as bifidobacteria using the genus-specific PCR primers according to Kok *et al.* [17]; the Bif164-PCR (5'-GGGTGGTAATGCCGGATG-3') and Bif662-PCR (5'-CCACCGTTACACCGGGAA-3') primers were used for specific amplification of *Bifidobacterium* 16S rDNA.

LPS-binding Assay

LPS-binding abilities of bifidobacteria were determined using a patented method [27]. Briefly, a stock solution of 1 mg/ml FITC-labeled LPS was prepared in distilled water. Bifidobacteria cells were washed twice with PBS (pH 7.4) and the cells were suspended in PBS at a final optical density (OD) of 0.4±0.01 at 600 nm, resulting in a density of approximately 10^7 cells/ml. The reaction mixture was prepared by adding FITC-labeled LPS to the cell suspensions to obtain concentrations ranging from 0 to 50 μg/ml. After incubation at 37°C for 30 min, cells were washed twice with PBS (pH 7.4) and then fixed in 1% paraformaldehyde at 4°C for 30 min. Flow cytometric analysis was performed by using a FACS caliber cytometer with blue-green excitation light (Becton-Dickinson, Basel, Switzerland).

Cell Surface Hydrophobicity Assay

The cell surface hydrophobicity of each bifidobacteria isolate was performed according to Lim *et al.* [18] and Pèrez *et al.* [24]. After washing, the bacterial cells were resuspended in PBS to an OD of 0.9±0.05 at 600 nm. Bacterial suspensions (3 ml) were vortexed with 1 ml of xylene for 2 min, and after allowing the phases to separate for 20 min, the absorbance of the aqueous phase was measured at 600 nm. CSH (%) was calculated using the absorbance value of the aqueous phase before and after mixing with xylene, according to the equation:

$$CSH\ (\%)=(A_i-A_f)/A_i\times 100$$

A_i, initial absorbance; A_f, final absorbance.

Table 1. LPS-binding activity, CSH, AA, and IL-8 release by the LPS-H and LPS-L groups.

Strain number	LPS-binding activity (%)	CSH (%)	AA (%)	Inhibition rate of IL-8 release (%)
LPS-high binding group (LPS-H)				
02	48 +/- 5.1	78	10	20
10	38 +/- 5.2	96	25	37
13	44 +/- 0.6	85	33	24
14	52 +/- 2.1	~	18	7
20	61 +/- 4.3	~	3	9
22	39 +/- 2.7	76	59	32
36	48 +/- 0.9	53	1	7
39	42 +/- 8.4	~	3	25
48	39 +/- 0.7	13	7	22
57	63 +/- 3.8	78	21	26
61	37 +/- 2.1	79	14	-14
LPS-low binding group (LPS-L)				
09	3 +/- 0.6	85	17	3
12	2 +/- 0.6	94	8	18
15	0 +/- 0.4	~	36	9
18	1 +/- 0.4	79	22	15
19	6 +/- 1.1	1	5	28
33	3 +/- 0.8	~	34	28
35	4 +/- 1.1	83	7	29
42	12 +/- 2.0	~	36	21
62	14 +/- 0.7	1	16	9
68	2 +/- 0.6	~	12	9

Results are expressed as mean +/- SD or mean only. *Determination of surface hydrophobicity was performed using the MATH method, following the method of Pérez *et al.* [24], and CSH<5% is marked by "~". Autoaggregation ability was measured according to Del Re *et al.* [7] and the value indicates the aggregative state 2 h after shaking.

Autoaggregation Assay

Autoaggregation (AA) was measured according to Del Re *et al.* [7] and expressed as a percentage. Briefly, bacterial cultures in stationary phase were shaken by vortexing and maintained at 15°C for 2 h. Samples (1 ml) of the upper suspension of the culture were then transferred to other tubes and the optical densities (OD 600 nm) were measured.

The AA percentage was calculated as

$$AA\ (\%)=\{1-(OD\ upper\ suspension\ /OD\ total\ bacterial\ suspension)\}\times 100.$$

IL-8 Assay

HT-29 (human colon adenocarcinoma) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and grown in a humidified incubator at 37°C in 5% CO_2, 95% air. HT-29 cells from passages 35–45 were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% antibiotics and seeded into 96-well plates at 3×10^4 cells/well. Bifidobacteria cells were washed twice with PBS (pH 7.4) and suspended in PBS to an OD of 0.4±0.01 at 600 nm (~10^7 cells/ml). The reaction mixtures were prepared by adding LPS stock solution to obtain LPS concentrations ranging from 0 to 50 μg/ml. After incubation at 37°C for 30 min, the reaction mixture was centrifuged at 10,000 ×*g* for 3 min, and 100 μl of the reaction mixture supernatant and LPS (1 μg/ml) were added to the HT-29 cells for 20 h. Cell culture medium was collected to determine the IL-8 content by ELISA (OptEIA, PharMingen, CA, U.S.A.).

Statistical Analysis

Statistical significance was analyzed using SAS (version 9.1). T-tests were performed to compare the LPS-H and LPS-L groups. The IL-8 assay was analyzed by a 1-way ANOVA

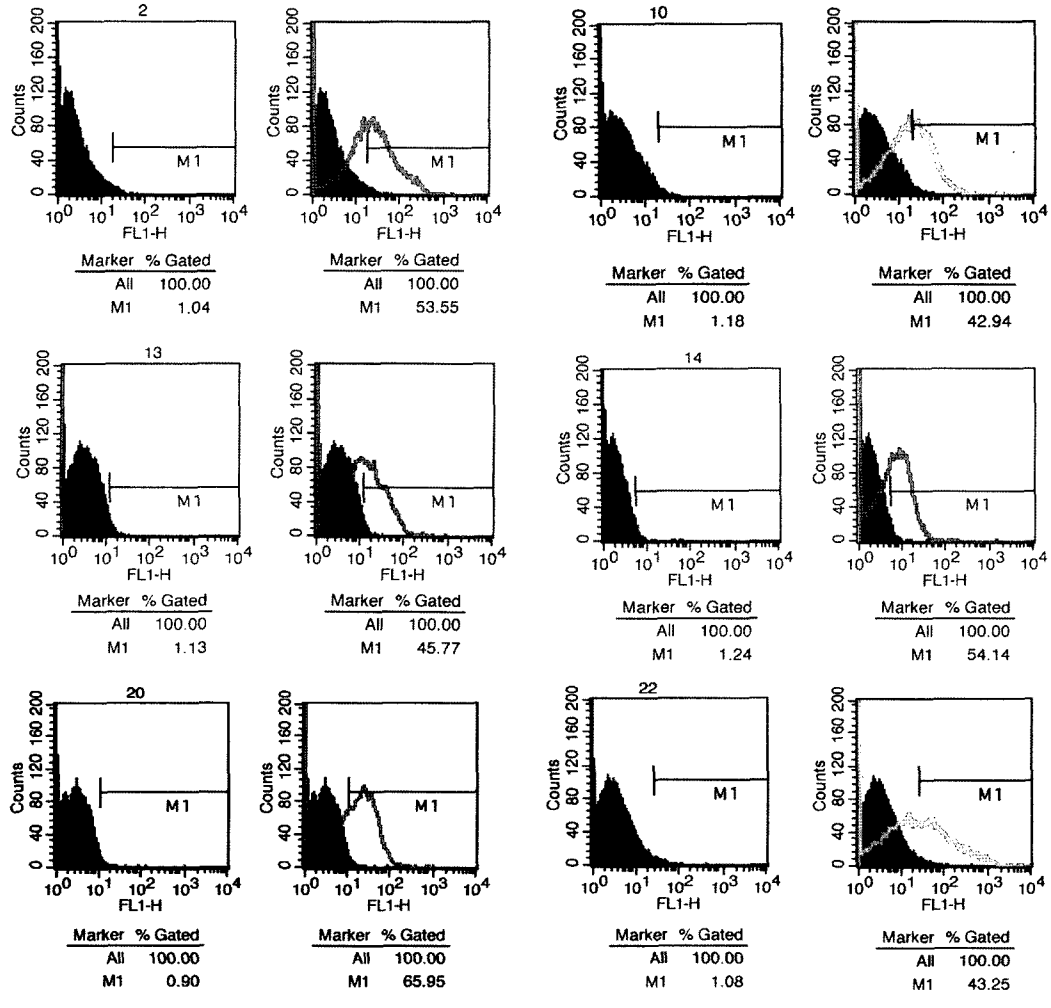

Fig. 1. FITC-labeled LPS-binding assay using an FACS caliber cytometer.

LPS-H and LPS-L strains were incubated with 50 μg/ml FITC-labeled LPS and separated by flow cytometry. LPS binding was detected as a shift in fluorescence intensity of some portion of the bifidobacterial population. In each pair of histograms, the panel on the left shows the untreated control population and the panel on the right shows the shift in fluorescence intensity in the LPS, treated population (gray line). At least 20,000 cells were scored for each strain. The strain number and LPS-binding ability (%) of each strain are presented at the top and bottom of each cytogram, respectively.

and Duncan's multiple range tests. $P<0.05$ was considered statistically significant.

RESULTS

Screening of Bifidobacteria with LPS-binding Ability

All the bifidobacterial strains tested were anaerobic, Gram-positive, nonspore-forming, pleomorphic rods, and had F6PPK activities and produced a common 523-bp PCR product in the genus-specific PCR [17] (data not shown). Ninety human bifidobacteria strains were investigated for their ability to bind *E. coli* LPS. In order to compare between LPS high and low binding groups, strains showing 30–60% binding were arbitrarily designated as LPS-high binding (LPS-H) and those with less than 15% binding were designated LPS-low binding (LPS-L) (Table 1). Examples of the FITC-labeled LPS-binding behavior of LPS-H and LPS-L strains are shown in Fig. 1.

Relationship Between LPS Binding, CSH, and Autoaggregation

CSH and AA properties of the experimental strains are shown in Table 1. Percentage autoaggregation of the LPS-H and LPS-L strains was plotted against hydrophobicity (data not shown). The CSH was highly variable among the strains. Seven strains (63.6%) in the LPS-H group and 4 strains (40%) in the LPS-L group showed over 50% CSH (Table 1). The LPS-binding ability and CSH % were not significantly correlated (t-test). This result shows that LPS binding is not directly related to CSH under the conditions used in this study.

Relationship Between LPS Binding, CSH, and Inhibition of IL-8 in HT-29 Cells

In this study, LPL-H and LPL-L bifidobacteria were examined for their ability to inhibit IL-8 release by HT-29 cells. Several bifidobacteria strains inhibited IL-8 release regardless of their LPS-binding ability or their CSH. Seven strains in the LPS-H group and 4 strains in the LPS-L group

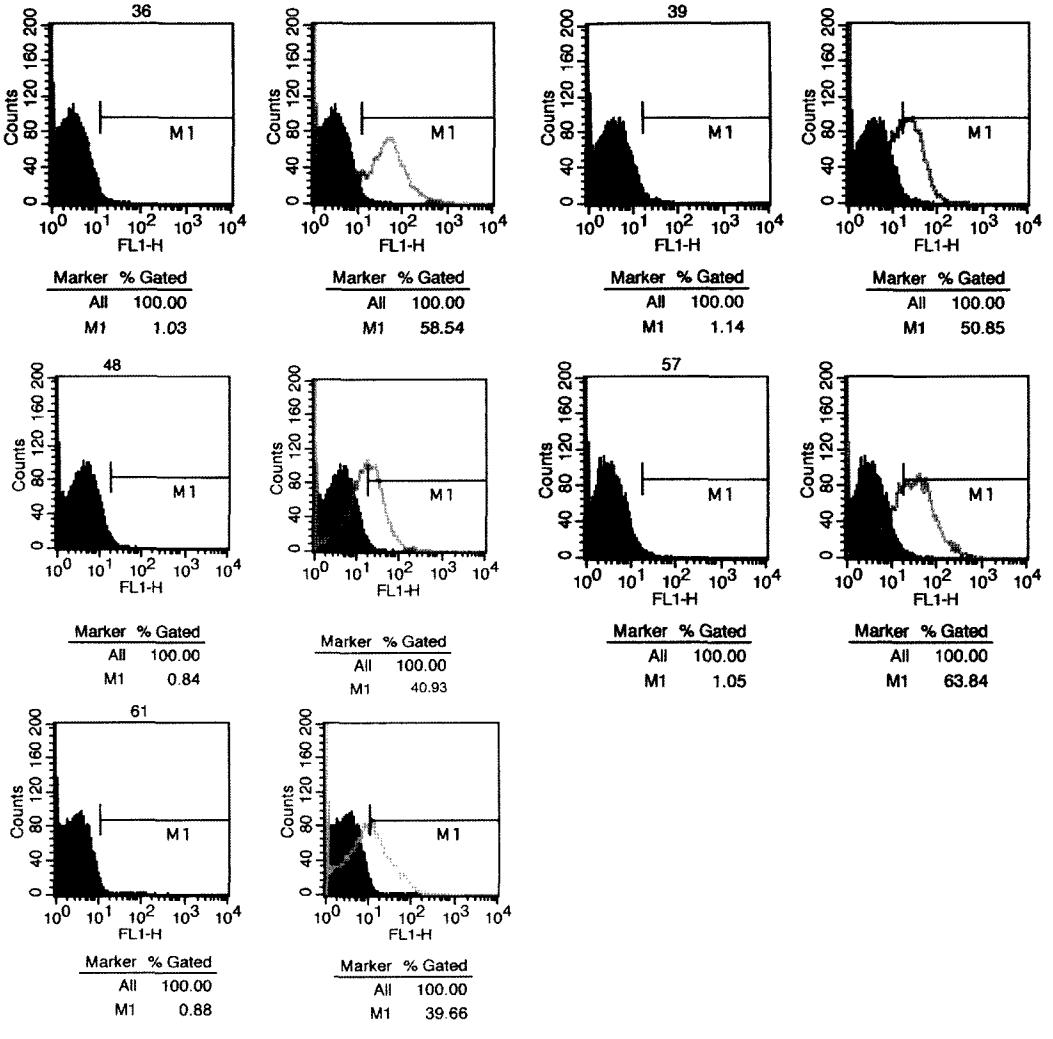

Fig. 1. Continued.

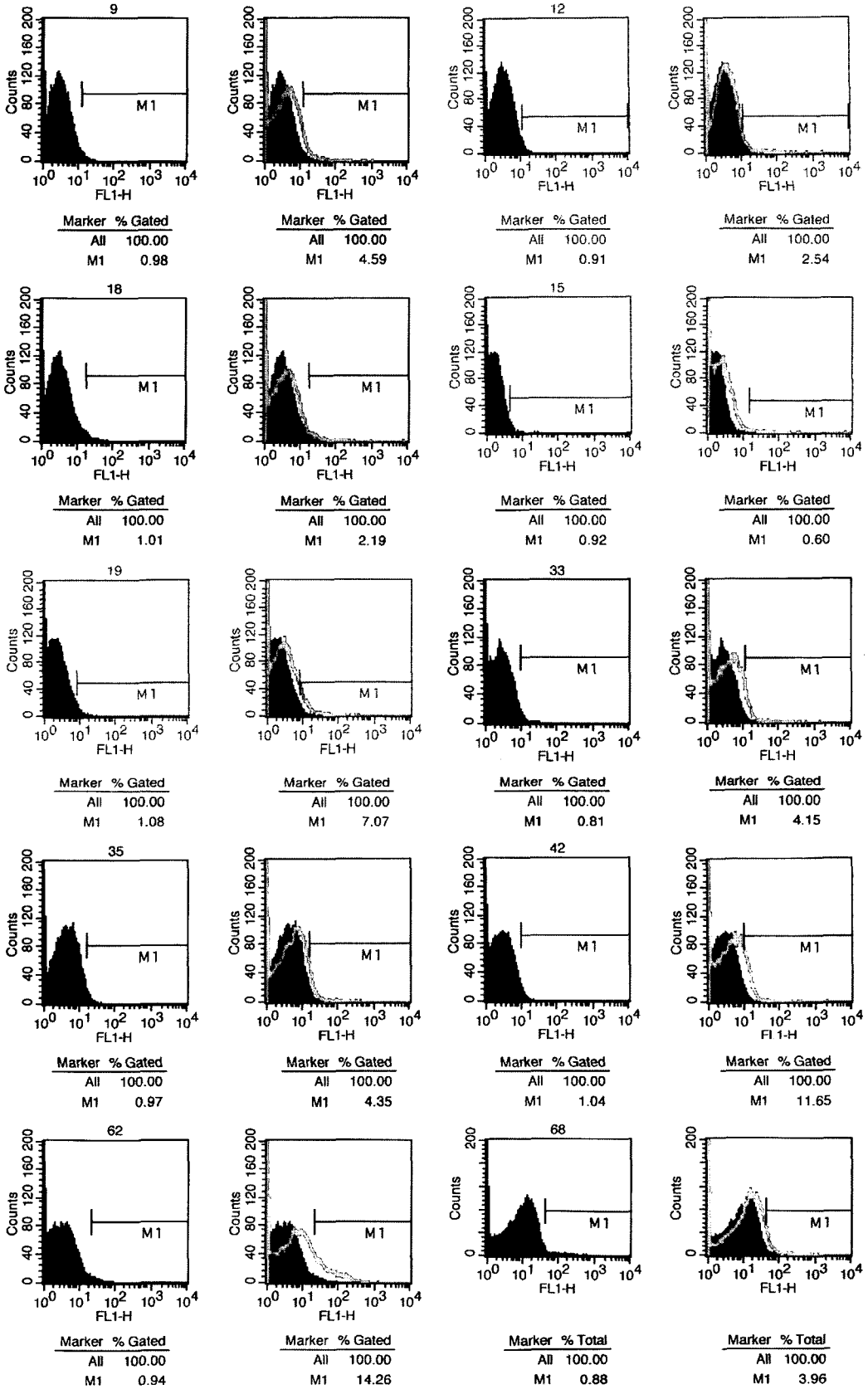

Fig. 1. Continued.

elicited greater than 20% inhibition of IL-8 production. Strains 02, 10, 13, 22, and 57, which showed high CSH and high LPS binding, significantly inhibited IL-8 release (Table 1). However, strains 12, 18, and 35, which showed low LPS binding but high CSH, still elicited a high degree of inhibition of IL-8 release (Table 1). The inhibition of IL-8 release by the LPS-H group tended to be greater than that of the LPS-L group, but the difference between groups was not significant (t-test). It is not clear whether LPS binding or CSH has an effect on the inhibition of LPS-induced IL-8 release by HT-29 cells. Therefore, it is important to note that the LPS-binding ability, CSH, AA, and inhibition of IL-8 by the individual bifidobacterial strains should be considered strain-dependent characteristics.

DISCUSSION

Previous studies have shown that the intestinal microflora is an important factor in the pathogenesis of IBD [28]. LPS plays as an important mediating factor for IBD by stimulating expression of TLR4 and CD14 in the intestinal epithelial cell [21, 22] and IBD patients [4]. Riedel *et al.* [25] showed that six out of eight bifidobacteria inhibited LPS-induced NF-κB activation in a dose- and strain-dependent manner in intestinal epithelial cells. They also showed that the physical presence of bifidobacteria cells during LPS challenge is required for their anti-inflammatory effects, indicating that this inhibition is not due to secreted compounds. Consequently, the direct interaction of bifidobacteria cells with LPS warranted further study. Adhesion of probiotic bacteria to the intestinal surface is considered to play a role in their colonization of the intestinal tract. Wadström *et al.* [30] reported a correlation between CSH and epithelial adhesion in lactobacilli.

However, some strains, in spite of their hydrophobic surface properties, were not capable of adhering to the intestinal epithelium, which suggested that multiple mechanisms are involved in the adhesion process. Recently, several studies have been performed to investigate the relationship between the CSH of bifidobacteria, their immunomodulating capacity, and interactions with pathogens in the gut. Some strains of *Bifidobacterium* spp. isolated from human intestine adhered to Caco-2 cells, probably as a result of their autoaggregation and CSH properties [8, 15]. Co-aggregative activity was detected between three strains of autoaggregative bifidobacteria and four autoaggregative strains of *Lactobacillus* spp. and two strains of enterohemorragic *E. coli* (O157). CSH was correlated with autoaggregating activity and adherence to mucin in *Lactobacillus*, but the correlation has not been confirmed in *Bifidobacterium* [12]. Likewise, in the present study, the CSH of bifidobacteria strains was highly variable and not closely correlated with LPS-binding ability or autoaggregation (Table 1). Additionally, the low correlation between CSH and autoaggregation differs from results obtained by other researchers [8, 24].

Vinderola *et al.* [29] found that all strains in their study interacted with sites of induction of the immune response in the gut. However, the corelation of the immunomodulatory activity with the CSH of the bacteria was not observed [14, 16]. Interleukin-8 (IL-8), one of the proinflammatory cytokines/chemokines produced in the intestine, initiates an acute inflammatory cascade and is an early marker of the inflammatory process. IL-8 expression must be controlled to prevent excessive tissue injury and damage both locally and distally [5].

Our study revealed that several bifidobacteria strains reduced IL-8 secretion regardless of their LPS-binding ability or hydrophobicity. For instance, strains 02, 10, 13, 22, and 57 possessed high CSH and LPS binding and significantly decreased IL-8 release. However, strains 12, 18, and 35 possessed low LPS binding and high CSH, but still inhibited IL-8 release. In fact, the inhibition of IL-8 release by the LPS-H group tended to be higher than that of the LPS-L group, but the difference was not significant and there was no correlation between the inhibition of IL-8 secretion and LPS binding or CSH among the experimental strains. Therefore, the LPS-binding ability, CSH, AA, and reduction in IL-8 secretion by bifidobacteria should be considered strain dependent characteristics. These results were further corroborated in our present results that some strains showing high LPS binding or inhibition of IL-8 secretion by HT-29 cells did not show high CSH. Further studies are required to determine how the ability to bind LPS affects the inhibition of IL-8 secretion from HT-29 with respect to the surface molecular properties of bifidobacteria. The ability of certain strains to downregulate the induction and release of IL-8 or bind LPS may have potential importance for modulating host gut immunomodulatory functions and could provide a basis for the prevention of endotoxic shock and sepsis of gut origin.

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