RESEARCH ARTICLE

Toll-like receptor-2-activating bifidobacteria strains differentially regulate inflammatory cytokines in the porcine intestinal epithelial cell culture system: finding new anti-inflammatory immunobiotics

Hitomi Fujie[1,2], Julio Villena[1,2], Masanori Tohno[3], Kyoko Morie[1,2], Tomoyuki Shimazu[1,2], Hisashi Aso[4], Yoshihito Suda[5], Takeshi Shimosato[6], Noriyuki Iwabuchi[7], Jin-Zhong Xiao[7], Tomoko Yaeshima[7], Keiji Iwatsuki[7], Tadao Saito[2], Muneo Numasaki[8] & Haruki Kitazawa[1,2]

[1]Food Immunology Group, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan; [2]Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan; [3]Functional Feed Research Team, National Institute of Livestock and Grassland Science, National Agricultural Research Organization, Tochigi, Japan; [4]Cell Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan; [5]Department of Food, Agriculture and Environment, Miyagi University, Sendai, Japan; [6]Fiber-Nanotech Young Researcher Empowerment Center, Shinshu University, Minamiminowa, Japan; [7]Food Science and Technology Institute, Morinaga Milk Industry Co. Ltd, Zama, Kanagawa, Japan; and [8]Department of Nutritional Physiology, Graduate School of Pharmaceutical Science, Josai University, Sakado, Japan

Correspondence: Haruki Kitazawa, Food Immunology Group, Graduate School of Agricultural Science, Tohoku University, Aobaku, Sendai 981-8555, Japan. Tel.: +81 22 717 8713; fax: +81 22 717 8715; e-mail: haruki@bios.tohoku.ac.jp

Present address: Hitomi Fujie, Department of Nutritional Physiology, Graduate School of Pharmaceutical Science, Josai University, Sakado 350-0295, Japan.

Received 22 November 2010; revised 12 June 2011; accepted 16 June 2011.
Final version published online 29 July 2011.

DOI:10.1111/j.1574-695X.2011.00837.x

Editor: Artur Ulmer

Keywords
anti-inflammatory bifidobacteria; porcine intestinal epithelila cells; TLR-2 activation; intestinal inflammation.

Abstract

A total of 23 strains of bifidobacteria taxonomically belonging to five species were tested for their potent immunomodulatory effect using a combination of two methods: the NF-κB-reporter assay using a toll-like receptor 2-expressing transfectant (HEK^{pTLR2} system) and the mitogenic assay using porcine Peyer's patches immunocompetent cells. Among the four preselected strains from different immunomodulatory groups, *Bifidobacterium breve* MCC-117 was able to efficiently modulate the inflammatory response triggered by enterotoxigenic *Escherichia coli* (ETEC) in a porcine intestinal epithelial (PIE) cell line. Moreover, using PIE cells and swine Peyer's patches immunocompetent cell co-culture system, we demonstrated that the immunoregulatory effect of *B. breve* MCC-117 was related to the capacity of the strain to influence PIE and immune cell interactions, leading to the stimulation of regulatory T cells. The results suggested that bifidobacteria that express high activity in both the HEK^{pTLR2} and the mitogenic assays may behave like potential anti-inflammatory strains. The combination of the HEK^{pTLR2} system, the evaluation of mitogenic activity and PIE cells will be of value for the development of new immunologically functional foods and feeds that could prevent inflammatory intestinal disorders. Although our findings should be proven in appropriate experiments *in vivo*, the results of the present work provide a scientific rationale for the use of *B. breve* MCC-117 to prevent ETEC-induced intestinal inflammation.

Introduction

Toll-like receptors (TLR) are major pattern recognition receptors (PRRs) in the induction of innate immunity through the recognition of exogenous microbial-associated molecular patterns (Lavelle *et al.*, 2010). One member of the TLR family, TLR2, is critical for the recognition of peptidoglycan, lipopeptides and lipoproteins of Gram-positive bacteria, mycoplasma lipopeptides or fungal zymosan (Takeda & Akira, 2005). Several works have demonstrated that TLR2 is required for some probiotics to exert their immunomodulatory effects. Studies in a primary culture of intestinal epithelial (IE) cells from conventional mice showed that immunostimulatory lactobacilli induce the release of cytokines by these cells via TLR2 (Vinderola *et al.*, 2005). In addition, lactobacilli are able to increase the expression of TLR2 in mice intestinal dendritic cells (DCs) and macrophages (Galdeano & Perdigón, 2006) and in human myeloid DCs (Mohamadzadeh *et al.*, 2005). Moreover, it was suggested that the strong induction of Th1-polarizing DCs by lactobacillus strains is dependent on intracellular nucleotide-binding oligomerization domain-2

sensing of peptidoglycan combined with a lipoteichoic acid interaction with TLR2 (Zeuthen *et al.*, 2008).

The majority of these studies were performed in rodent experimental models; however, in recent years, there has been a growing interest in the porcine immune system because of its potential as a model for the study of the human immune system (Bailey, 2009). In this sense, we isolated the porcine TLR2 (pTLR2) cDNA from ileal Peyer's patches and, using a human cell line, we developed a method for evaluating the immune responses to immunobiotic microorganisms by constructing a pTLR2-expressing transfectant (HEK^{pTLR2} cells) (Tohno *et al.*, 2007b; Kitazawa *et al.*, 2008). We used the HEK^{pTLR2} immunoassay system to evaluate various lactobacilli and to select among them the immunoenhancing strains and we found that immunostimulatory strains with increased ability to stimulate the activity of NF-κB via pTLR2 were also the most effective to polarize a Th1-mediated immune response. Our studies demonstrated that immunobiotic lactobacilli can be successfully screened using this system and that the different immunomodulatory activities of various strains can be accurately detected by HEK^{pTLR2} cells (Tohno *et al.*, 2007b; Kitazawa *et al.*, 2008).

On the other hand, it has been demonstrated that TLR2 seems to play an important regulatory role in the recognition of bifidobacteria that possess an immunoinhibitory effect. Hoarau *et al.* (2006) found that the supernatant of *Bifidobacterium breve* C50 can induce DCs maturation and prolonged survival through TLR2, with a high interleukin (IL)-10 production. Bifidobacteria have been shown to inhibit the production of tumor necrosis factor (TNF)-α and IL-6 induced by immunostimulatory lactobacilli in blood immune cells via interaction with TLR2 (Zeuthen *et al.*, 2008). In addition, it was demonstrated, using bone marrow-derived DCs from $TLR2^{-/-}$ mice, that bifidobacteria induced much higher levels of IL-12 and lower IL-10 levels in DCs that are $TLR2^{-/-}$ when compared with wild-type DCs (Zeuthen *et al.*, 2008). These studies demonstrate that TLR2 is not only involved in the immunoenhancing activity of lactobacilli, but may also be involved in the immunoregulatory activity of bifidobacteria.

Considering these antecedents, the aim of this study was to evaluate whether the HEK^{pTLR2} immunoassay system, which is useful for the selection of immunoenhancing lactobacilli strains (Tohno *et al.*, 2007b), could also be used to select immunobiotic bifidobacteria strains, which possess immunoregulatory/anti-inflammatory capacity. Moreover, we attempted to select, using the combination of the HEK^{pTLR2} immunoassay system and the mitogenic assay using porcine immunocompetent cells from Peyer's patches, certain immunobiotic bifidobacteria able to efficiently modulate the inflammatory response triggered by enterotoxigenic *Escherichia coli* (ETEC) in porcine intestinal epithelial (PIE) cells.

Materials and methods

Bacteria

Bifidobacteria were provided by Morinaga Milk Industry Co. Ltd (Zama, Japan). Twenty-three different strains were used in the experiments: *Bifidobacterium longum* strains BB536, ATCC15707T, ATCC15708, MCC-50, MCC-274, MCC-1083; *Bifidobacterium infantis* strains M-63, MCC-10, ATCC15700T, MCC-1021; *Bifidobacterium bifidum* strains ATCC15696, MCC-135, MCC-348, MCC-1092; *Bifidobacterium adolescentis* strains ATCC15706, ATCC15705, MCC-75, MCC-1138, IV-11; and *B. breve* strains MCC-117, MCC-121, M-16V, MCC-272, MCC-1093. Bifidobacteria were grown in Man-Rogosa-Sharpe broth and agar (Difco, Detroit, MI) supplemented with 0.05% (w/v) cysteine (Sigma, Tokyo, Japan), and incubated at 37 °C for 16 h under anaerobic conditions (AnaeroGen; Oxoid, Basingstoke, UK). Cultures were then centrifuged at 1900 ***g*** for 10 min and bifidobacteria were washed with phosphate-buffered saline (PBS) and resuspended in DMEM cell culture medium at the appropriate concentrations. ETEC strain 987P was kindly provided by Dr M. Nakazawa, National Institute of Animal Health (Tsukuba, Japan). ETEC cells were plated into tryptic soy agar (Becton, Dickinson and Company) supplemented with 5% sheep blood (Nippon Biotest Laboratories Inc., Tokyo, Japan). After an overnight incubation at 37 °C, a single colony was transferred to tryptic soy broth and grown for 24 h at 37 °C with shaking (200 r.p.m.). After overnight incubation, the subcultures of bacteria that had been grown until the mid-log phase were centrifuged at 1900 ***g*** for 10 min at 4 °C and washed with PBS, heat-treated at 65 °C for 30 min and resuspended in DMEM cell culture medium.

Mitogenicity assay

Porcine Peyer's patches immunocompetent cells were placed in a 96-well microplate (Costar, 2×10^5 cells per well) and incubated at 5% CO_2, 37 °C, for 48 h in a complete RPMI 1640 medium (Sigma) supplemented with 2% fetal bovine serum (FCS). Immune cells were stimulated with bifidobacteria (10 μg per well) for 48 h. In the final hour of culture, the cells were radiolabeled with 9.25 kBq per well of [methyl-3H]-uridine (GE Healthcare, Tokyo, Japan). The cells were then harvested with a glass fiber filter (PerkinElmer Japan, Kanagawa, Japan). The [methyl-3H]-uridine incorporation was counted in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA). The results are presented as the SI, calculated using the following equation:

$$[(\text{counts per minute in treated cultures}) - (\text{counts per minute in background})] / [(\text{counts per minute in control cultures}) - (\text{counts per minute in background})]$$

NF-κB reporter luciferase assay

The human embryonic kidney cell line, HEK293, was obtained from the TKG cell bank (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) and transfected with a plasmid encoding pTLR2 (HEK^{pTLR2} cells) as described previously (Tohno *et al.*, 2007b). HEK^{pTLR2} cells were maintained in DMEM (Sigma) supplemented with 10% FCS (Hyclone, Logan, UT). The NF-κB reporter assay was performed as described previously (Tohno *et al.*, 2007a, b). HEK^{pTLR2} cells were then stimulated with the different bifidobacteria strains (50 mg per well) for 24 h and the luciferase assays were performed according to the manufacturer's protocol (Promega, Tokyo, Japan). The relative index (RI) was estimated from the resonance units (RU) using the following equation:

$$\mathrm{RI} = [(\mathrm{RU} \times \mathrm{HEK}^{\mathrm{pTLR2}}) - (\mathrm{RU\ in\ background})] / [(\mathrm{RU\ in\ HEK\ control}) - (\mathrm{RU\ in\ background})]$$

All assays were conducted at least three times in triplicate wells.

Evaluation of the anti-inflammatory activity of bifidobacteria in PIE cells

PIE cells, which are nontransformed intestinal cell lines originally derived from intestinal epithelia isolated from an unsuckled neonatal swine, were isolated and cloned previously (Moue *et al.*, 2008). For the passage, PIE cells were treated with a sucrose/EDTA buffer for 4 min, detached using 0.04% trypsin in PBS and then plated at 1.5×10^4 cells cm^{-2} in culture flasks (Nalge Nunc International, Rochester, NY) at 37 °C in an atmosphere of 5% CO_2. PIE cells were cultured in 10% FCS DMEM and passaged every 3 or 4 days. For the evaluation of the anti-inflammatory activity of bifidobacteria, PIE cells were seeded at 3×10^4 cells per well in a collagen-coated 12-well multiplate (Sumitomo Bakelite Co. Ltd, Japan). PIE cells were stimulated with bifidobacteria (100 multiplicity of infection: MOI) for 48 h and then washed twice with PBS. Finally, PIE cells were stimulated with ETEC (100 MOI) for 12 h. Culture was stopped using the TRIzol Reagent (Invitrogen, Life Technologies). Studies of mRNA and protein expression of different cytokines were performed as described below.

Evaluation of the anti-inflammatory activity of bifidobacteria in PIE-immunocompetent cells co-culture system

Porcine Peyer's patches immunocompetent single-cell suspensions were prepared from the ileum of adult swine as described previously (Shimosato *et al.*, 2005a; Tohno *et al.*, 2005, 2006). All procedures were conducted in accordance with the Guidelines for Animal Experimentation of Tohoku University. In the Transwell culture system, PIE cells were seeded in the apical surface at a concentration of 1.5×10^5 cells per well in a 12-well tissue culture plate [Transwell-Col. (PTFE), pore size 0.2 μm] while porcine Peyer's patches immunocompetent cells were seeded in the apical surface at a concentration of 2×10^7 cells per well. For the evaluation of the anti-inflammatory activity of bifidobacteria in the PIE-immunocompetent cells co-culture system, the apical surface containing PIE cells was stimulated with bifidobacteria (100 MOI) for 48 h and then washed twice with PBS. Finally, PIE cells were stimulated with ETEC (100 MOI) for 12 h. Culture was stopped using the TRIzol Reagent. Studies of mRNA and protein expression of different cytokines were performed as described below.

Quantitative expression analysis by real-time PCR (RT-PCR)

To quantify cytokine mRNA using RT-PCR, cDNA standards were produced for each cytokine, and β-actin as described previously (Moue *et al.*, 2008). RT-PCR was carried out using a 7300 Real-time PCR System (Applied Biosystems, Warrington, UK) using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen) and the primers for β-actin (sense: TGG ATA AGC TGC AGT CAC AG; antisense: GCG TAG AGG TCC TTC CTG ATG T); IL-6 (sense: AGA ATC TCA GAA ACC CGA CTG TTT; antisense: ATT ATC CGA ATG GCC CTC AG) and IL-8 (sense: GCT CTC TGT GAG GCT GCA GTT; antisense: TTT ATG CAC TGG CAT CGA AGT T). Sequencing confirmed that the amplified cDNA was a fragment of the targeted cytokine or chemokine. Each reaction was performed in triplicate.

Flow cytometric analysis

Expression levels of cytokine and chemokine proteins in PIE cells and porcine Peyer's patches immunocompetent cells were determined by flow cytometry as described previously (Shimosato *et al.*, 2005b). PIE cells were labeled with primary antibodies: anti-pig IL-6 mouse IgG2b (R&D Systems) or anti-pig IL-8 mouse IgG2a (Abcam), followed by secondary antibodies: anti-mouse IgG2b-FITC or anti-mouse IgG2a-PerCP (Southern Biotech) for the detection of IL-6 and IL-8. Immune cells were labeled with the following primary antibodies: anti-pig CD3e biotin-conjugated (eBioscience), anti-pig CD8 biotin-conjugated (eBioscience), anti-pig CD25 mouse IgG1 (Southern Biotech), anti-pig CD4 FITC-conjugated (Santa Cruz Biotechnology), anti-pig IL-1β mouse IgG1 (R&D Systems), anti-pig IL-2 mouse IgG2b (R&D Systems), anti-pig IL-6 mouse IgG2b (R&D Systems), anti-pig IL-8 mouse IgG2a (Abcam), anti-pig IL-10 mouse IgG2b (R&D Systems) or anti-pig IFN-γ mouse IgG2b (Abcam). In addition, streptavidin FITC- and PE-Cy5-conjugated (eBioscience), anti-mouse IgG1-FITC (Santa Cruz Biotechnology), anti-mouse IgG2b-PE, anti-mouse IgG2a-PE and anti-mouse IgG1-PE (Santa

Cruz Biotechnology) were used as secondary antibodies. Analysis was performed using FACS-Calibur™ (BD, Franklin Lakes, NJ) and FLOWJO software (Tree star, Ashland, OR).

Statistical analysis

Statistical analysis was performed using GLM and REG procedures of SAS computer program. The comparisons among the mean values of the relative mRNA expressions of inflammatory cytokines, the NF-κB activity and the mitogenic activity in cells stimulated by each bifidobacteria strain were carried out using one-way ANOVA and Fisher's least significant difference tests. For these analyses, $P < 0.05$ level was used to define significance. The correlation between the NF-κB and the mitogenic activities was shown by a linear regression function and coefficient of determination. The discriminant analysis was performed to distinguish the anti-inflammatory ability of each bifidobacteria strains from the control by summarizing the multiple information obtained with cytokine mRNA expressions studies. The discriminant indexes were calculated by fitting their multi information to a discriminant function. Then, discriminant scores were calculated by summarizing cytokine information by fitting relative mRNA expressions in cells by stimulation of each strain to a linear discriminant function, and the ratio possible to discriminate each strain by cytokine mRNA expressions in cells was predicted.

Results

Screening of bifidobacteria strains

Of the 23 strains studied, 11 were able to increase NF-κB activity in relation to the control group (Fig. 1a). Within the

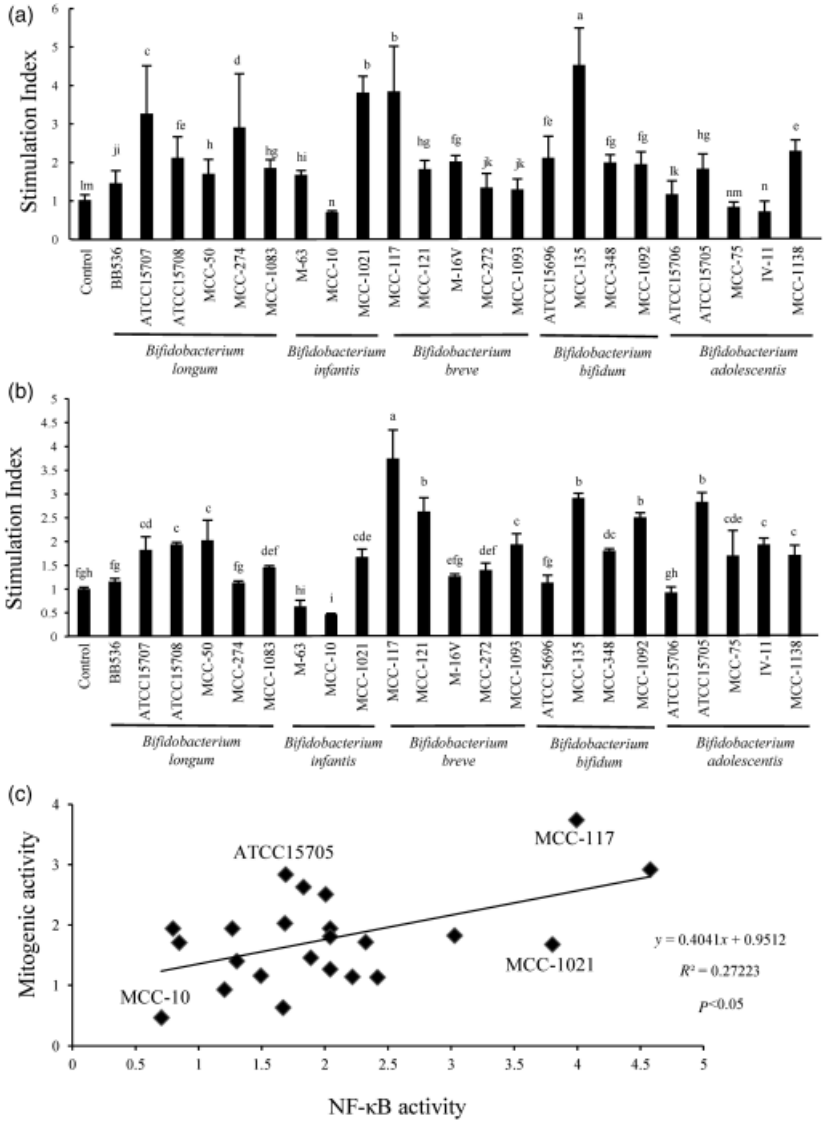

Fig. 1. Selection of immunomodulatory bifidobacteria strains by (a) HEK^{pTLR2} immunoassay system and (b) the mitogenic assay. Values represent the means and error bars indicate the SD. The results are representative of three or four independent experiments. Values with different superscripts letters are significantly different within the level $P < 0.05$. (c) Correlation between the HEK^{pTLR2} immunoassay system and the mitogenic assay by a linear regression function and coefficient of determination.

group of bacteria with a stimulating effect, we found representatives of the five species of bifidobacteria used in this study. However, *B. infantis* MCC-1021, *B. breve* MCC-117 and *B. bifidum* MCC-135 showed an effect superior to the other strains with a stimulating capacity. Another method commonly used to study the immunostimulatory properties of potential probiotic microorganisms is the evaluation of mitogenic activity. We used this method to assess the immunostimulatory capacity of the 23 strains of bifidobacteria, using immunocompetent cells isolated from swine Peyer's patches. Fourteen of the strains, belonging to the five species studied, were able to significantly increase the mitogenic activity compared with the control group (Fig. 1b). However, the effect of MCC-117, MCC-121, MCC-135, MCC-1092 and ATCC15705 strains was higher than that observed in the other strains with a stimulating capacity. On the contrary, *B. infantis* MCC-10 was the only strain that strongly decreased mitogenic activity (Fig. 1b). Considering the results obtained using the two screening methods and the analysis of the correlation between both the methods by a linear regression function and coefficient of determination (Fig. 1c), bifidobacteria strains with a potential immunomodulatory effect in our study can be classified into four groups: (1) strains with a high stimulatory capacity of HEK^{pTLR2} cells and high mitogenic activity, such as *B. breve* MCC-117 and *B. bifidum* MCC-135; (2) strains with a high stimulatory capacity of HEK^{pTLR2} cells and moderate mitogenic activity, such as *B. infantis* MCC-1021; (3) strains unable to stimulate HEK^{pTLR2}, but with a high mitogenic activity, such as *B. adolescentis* ATCC15705; and (4) strain unable to stimulate HEK^{pTLR2} and with the capacity to suppress mitogenic activity, such as *B. infantis* MCC-10. For this reason, we selected *B. breve* MCC-117, *B. infantis* MCC-1021, *B. adolescentis* ATCC15705 and *B. infantis* MCC-10 for further studies.

Anti-inflammatory activity of bifidobacteria on PIE cells

The stimulation of PIE cells with ETEC significantly increased the mRNA levels of IL-6, IL-8 and MCP-1 (Fig. 2a–c). When the PIE cells were stimulated previously with bifidobacteria strains, the response of PIE cells to the ETEC challenge was significantly different from that observed in the control group. The mRNA levels of IL-6, IL-8 and MCP-1 in PIE cells stimulated with *B. breve* MCC-117, *B. infantis* MCC-1021, *B. adolescentis* ATCC15705 and *B. infantis* MCC-10 were lower than those observed in the

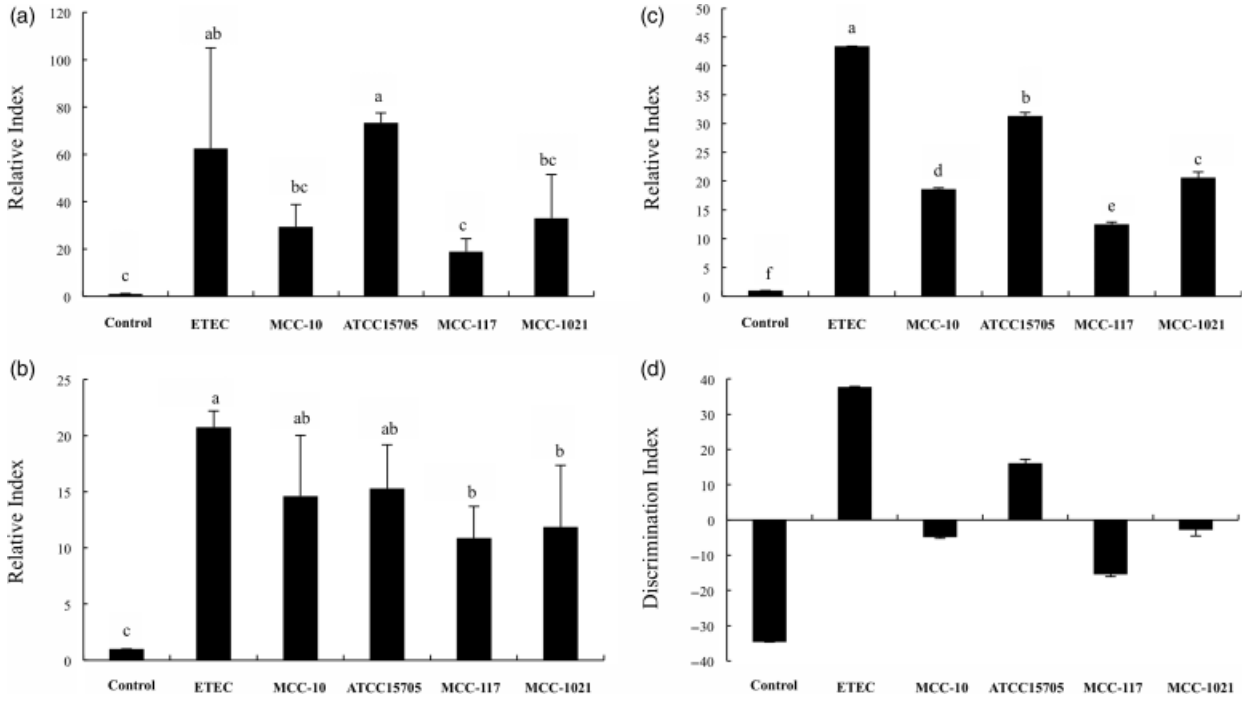

Fig. 2. Expression of (a) IL-6, (b) IL-8 and (c) MCP-1 mRNA on PIE cells after inflammatory stimulation. PIE cells were pretreated with bifidobacteria (MCC-10, ATCC15705, MCC-117 or MCC-1021 strains) for 48 h and then stimulated with ETEC for 12 h. PIE cells without previous treatment (ETEC group) and PIE cells without ETEC challenge (control group) were used as controls. Values represent the means and error bars indicate the SD. The results are representative of three or four independent experiments. Values with different superscripts letters are significantly different within the level $P < 0.05$. (d) Discriminant analysis of inflammatory cytokine expression on PIE cells.

control. The four strains were equally effective in reducing the levels of IL-8, while the levels of IL-6 and MCP-1 in PIE cells treated with *B. breve* MCC-117 were significantly lower than those found in cells treated with the other bifidobacteria strains (Fig. 2a–c).

In order to define the characteristic immunomodulatory ability of each bifidobacteria strain, their anti-inflammatory activities were studied by discriminant analysis. Cytokines mRNA levels were considered as values to discriminate and a function was predicted as $y = 0.0382x_1 + 0.0328x_2 + 0.9971x_3$, considering that y was the discriminant score and x_1, x_2 and x_3 were IL-6, IL-8 and MCP-1 mRNA expression levels, respectively. As showed in Fig. 2d, the discriminant analysis indicated that each of the four strains has its own immunomodulatory capacity and that the strains with the highest and the lowest anti-inflammatory activities are MCC-117 and ATCC15705, respectively. Thus, these two strains were selected for further studies.

Immunomodulatory activity of bifidobacteria in an *in vitro* Peyer's patch model culture system

The study of the expression of IL-6 and IL-8 proteins on PIE cells revealed that the levels of both cytokines increased significantly after the challenge with ETEC in all experimental groups (Fig. 3). The PIE cells stimulated with ETEC presented significantly higher levels of IL-6 and IL-8 than those observed in the control group (Fig. 3). However, the PIE cells pretreated with *B. breve* MCC-117 reduced IL-6 and IL-8 production, while those effects were not observed in *B. adolescentis* ATCC15705 (Fig. 3). In the $CD4^-CD8^-$ population, we studied the expression of IL-1β and IL-6, two cytokines produced by the antigen-presenting cells (APCs) that are important in T-cell activation. ETEC challenge did not modify the expression of IL-1β or IL-6 in Peyer's patches $CD4^-CD8^-$ cells (Fig. 4). Pretreatment with *B. adolescentis* ATCC15705 or *B. breve* MCC-117 did not induce changes in the levels of IL-1β produced by PIE cells in response to ETEC stimulation (Fig. 4). However, the levels of IL-6 were significantly lower in $CD4^-CD8^-$ prestimulated with the bifidobacteria strains (Fig. 4). The levels of IL-2 in the $CD4^+CD8^-$ and $CD4^-CD8^+$ populations were not modified by ETEC stimulation (Fig. 5). However, there were increased levels of IFN-γ in both effector T cells and cytotoxic T lymphocytes in the ETEC control group (Fig. 5). $CD4^+$ and $CD8^+$ lymphocytes in the *B. breve* MCC-117 group presented levels of IFN-γ that were significantly lower than the ETEC control, whereas the levels of this cytokine in the *B. adolescentis* ATCC15705 group were similar to the ETEC control (Fig. 5).

Finally, we studied the expression of IL-10 in $CD4^+CD25^{high}$ regulatory T (Treg) cells. ETEC decrease the expression of IL-10 in Treg cells in the ETEC control group

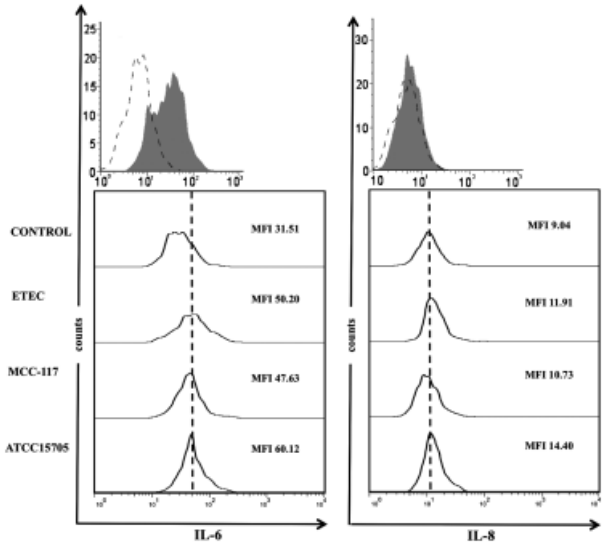

Fig. 3. Expression of IL-6 and IL-8 proteins on PIE cells after inflammatory stimulation. PIE cells were co-cultured with porcine Peyer's patches immunocompetent cells, pretreated with bifidobacteria (ATCC15705 or MCC-117 strains) for 48 h and then stimulated with ETEC for 12 h. PIE cells without previous treatment (ETEC group) and PIE cells without ETEC challenge (control group) were used as controls. Histograms show flow cytometric analysis as follows: PIE cells stained with anti-IL-8 or anti-IL-6 antibodies (bold lines), isotype-matched controls (dotted lines) and permeabilization controls (solid histograms). Values of mean fluorescence intensity (MFI) are showed for each group.

(Fig. 6). On the contrary, $CD4^+CD25^{high}$ Treg lymphocytes of bifidobacteria-treated groups presented levels of IL-10 that were significantly higher than those of the ETEC control, the effect of *B. breve* MCC-117 being superior than that of *B. adolescentis* ATCC15705 (Fig. 6).

Discussion

TLR2 is one of the PRRs that would be of great importance for the immunomodulatory effect of probiotic microorganisms. It has been suggested that lactobacilli and bifidobacteria act as immunostimulators and immunoregulators, respectively, through the interaction with TLR2 (Mohamadzadeh *et al.*, 2005; Vinderola *et al.*, 2005; Galdeano & Perdigón, 2006; Hoarau *et al.*, 2006; Zeuthen *et al.*, 2008). In the present study, we examined the effect of 23 bifidobacteria strains, belonging to different species, on the activation pattern of TLR2-overexpressing cells. As it has been observed in studies in which bifidobacteria were evaluated using immune cells (López *et al.*, 2010), we observed a strain-specific capacity of bifidobacteria to stimulate HEK^{pTLR2} cells. Of the strains tested, *B. infantis* MCC-1021, *B. breve* MCC-117 and *B. bifidum* MCC-135 were the ones that showed the highest NF-êB activity in the HEK^{pTLR2} immunoassay system, while other strains of the same species were less effective or did not modify this parameter.

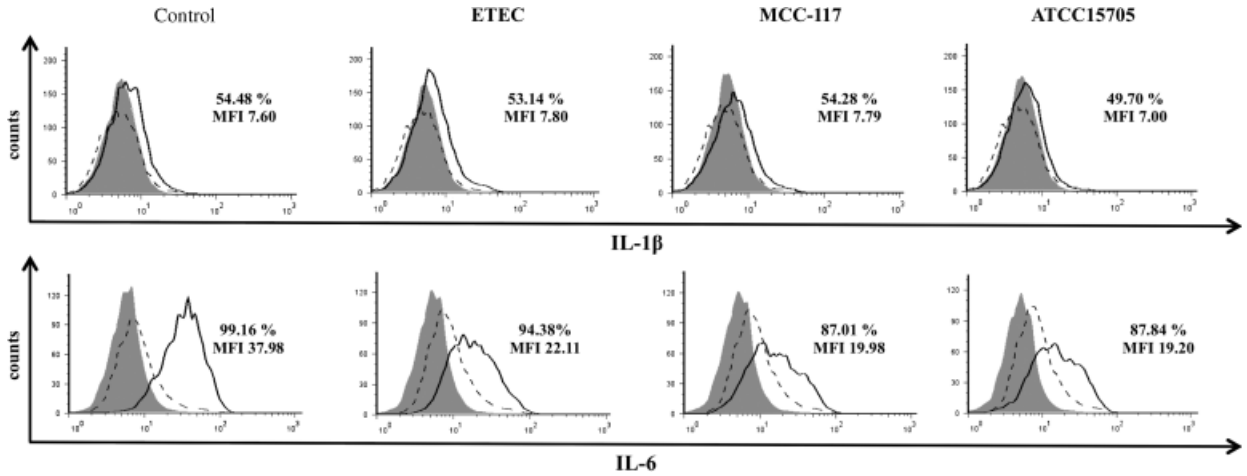

Fig. 4. Expression of IL-1β and IL-6 proteins on $CD4^{-}CD8^{-}$ cells after inflammatory stimulation. PIE cells were co-cultured with porcine Peyer's patches immunocompetent cells, pretreated with bifidobacteria (ATCC15705 or MCC-117 strains) for 48 h and then stimulated with ETEC for 12 h. PIE cells without previous treatment (ETEC group) and PIE cells without ETEC challenge (control group) were used as controls. Histograms show flow cytometric analysis as follows: $CD4^{-}CD8^{-}$ cells stained with anti-IL-1β or anti-IL-6 antibodies (bold lines), isotype-matched controls (dotted lines) and permeabilization controls (solid histograms). Values of mean fluorescence intensity (MFI) are shown for each group.

Mitogenic activities are generally considered to be one of the most important properties of immune modulators, and have been used to evaluate the immunomodulating activities of lactic acid bacteria (Kitazawa *et al.*, 1992; He *et al.*, 2006). In this work, we used this methodology to evaluate the same strains of bifidobacteria and to assess whether a correlation exists between the two methods for the selection of probiotics. As observed with the HEK^{pTLR2} system, the results showed a difference in the mitogenic activity between the different bifidobacteria strains, despite their genetic similarity. Thus, in order to determine the relation among NF-κB and mitogenic activities, we next selected four strains from each group classified by the correlation of the two screening methods and we evaluated their anti-inflammatory effect in PIE cells challenged with an intestinal pathogen. PIE cells are able to induce inflammatory responses by upregulating cytokines and chemokines in response to lipopolysaccharide, suggesting that PIE cells are useful cell lines for studying inflammatory responses (Moue *et al.*, 2008). In preliminary experiments, we observed that the stimulation of PIE cells with porcine-specific ETEC significantly increased the mRNA levels of IL-6, IL-8 and MCP-1 at hour 12 after the challenge (unpublished data). This is consistent with our previous results (Moue *et al.*, 2008) and with the results from another group that demonstrated that infection of human intestinal Caco-2 cells with ETEC caused a strong upregulation of proinflammatory cytokines and chemokines (Roselli *et al.*, 2006). When PIE cells were stimulated with the four selected strains before the challenge with ETEC, the mRNA levels of IL-6, IL-8 and MCP-1 in almost every treatments were lower than those observed in the ETEC control group, even though the response of PIE cells was different for each of the four strains. We proved that each selected strain has a specific immunomodulatory characteristic and that the difference in NF-κB and mitogenic activities' stimulation is related to the anti-inflammatory property of bifidobacteria. Interestingly, *B. breve* MCC-117 presented the most remarkable anti-inflammatory effect among the four selected strains. Thereby, bifidobacteria that express high activity in both the HEK^{pTLR2} immunoassay system and the mitogenic assay may be a potential immunobiotic bifidobacteria strain with high anti-inflammatory activity.

Although the study in PIE cells demonstrated the ability of *B. breve* MCC-117 to modulate the inflammatory response, this *in vitro* model is simplified and may neglect the effect of cell–cell interactions in a complex organic microenvironment, which completely changes the resulting response. IE cells express a broad range of factors that may influence intestinal APCs and lymphocytes (Rescigno *et al.*, 2008; Westendorf *et al.*, 2010). In the steady state, IE cells create a tolerogenic environment that favors the promotion and development of tolerogenic APCs and $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells (Rimoldi *et al.*, 2005; Westendorf *et al.*, 2010). However, in the presence of pathogenic bacteria, IE cells function as APCs to different subsets of T cells (Hershberg & Mayer, 2000) and, moreover, through the secretion of IL-1β, IL-6, IL-8, IL-18 and TNF-α, play a role in the activation of innate immune response (Stadnyk, 1994; Rimoldi *et al.*, 2004; Moue *et al.*, 2008; Blumberg, 2009). Thus, together with local immune cells, it is the intestinal epithelium that governs the induction of oral tolerance or

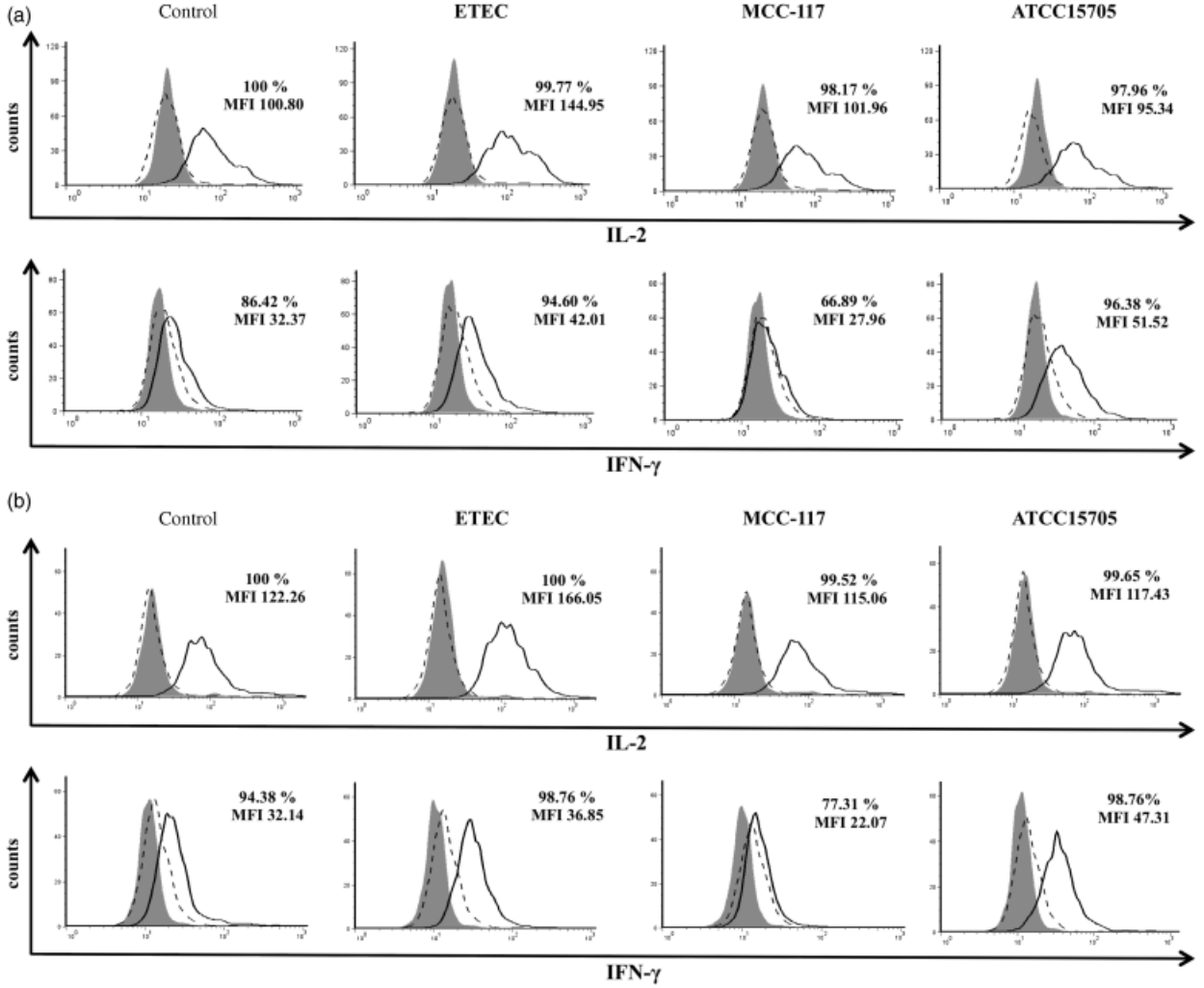

Fig. 5. Expression of IL-2 and IFN-γ proteins on (a) $CD4^+CD8^-$ and (b) $CD4^-CD8^+$ cells after inflammatory stimulation. PIE cells were co-cultured with porcine Peyer's patches immunocompetent cells, pretreated with bifidobacteria (ATCC15705 or MCC-117 strains) for 48 h and then stimulated with ETEC for 12 h. PIE cells without previous treatment (ETEC group) and PIE cells without ETEC challenge (control group) were used as controls. Histograms show flow cytometric analysis as follows: $CD4^+CD8^-$ and $CD4^-CD8^+$ cells stained with anti-IL-2 or anti-IFN-γ antibodies (bold lines), isotype-matched controls (dotted lines) and permeabilization controls (solid histograms). Values of the mean fluorescence intensity (MFI) and percentage of positive cells are shown for each group.

inflammation. Thereby, to further assess the immunomodulatory effect of *B. breve* MCC-117, we used a co-culture system with a PIE cell monolayer and immunocompetent cells from porcine Peyer's patches, modeling an *in vitro* Peyer's patches culture system. The results confirmed that the challenge of PIE cells with ETEC induced a significant increase in the production of IL-6 and IL-8 by these cells. However, unlike the observation at hour 12 post-ETEC challenge, at hour 36, PIE cells stimulated with bifidobacteria in the co-culture system presented higher IL-6 levels than the control group. Increased production of IL-6 in IE cells induced by probiotic microorganisms has been described previously (Vinderola *et al.*, 2005). It is known that IL-6 is able to favor the clonal expansion of IgA B lymphocytes, increasing the number of IgA-producing cells in the lamina propria of the gut. IgA is important to avoid the colonization of mucosal tissues and the subsequent spreading of pathogens. This protective effect reduces inflammatory reactions and prevents potentially harmful effects on the tissue (Villena *et al.*, 2005; Alvarez *et al.*, 2009; Salva *et al.*, 2010).

Taking into account that cytokine production and release by IE cells may modify the activity of immune cells (Stadnyk, 1994; Rimoldi *et al.*, 2004; Blumberg, 2009), we next evaluated the changes induced in the APCs, represented in our study by the $CD4^-CD8^-$ cells population. The production of IL-6 by APCs during infection appears to be

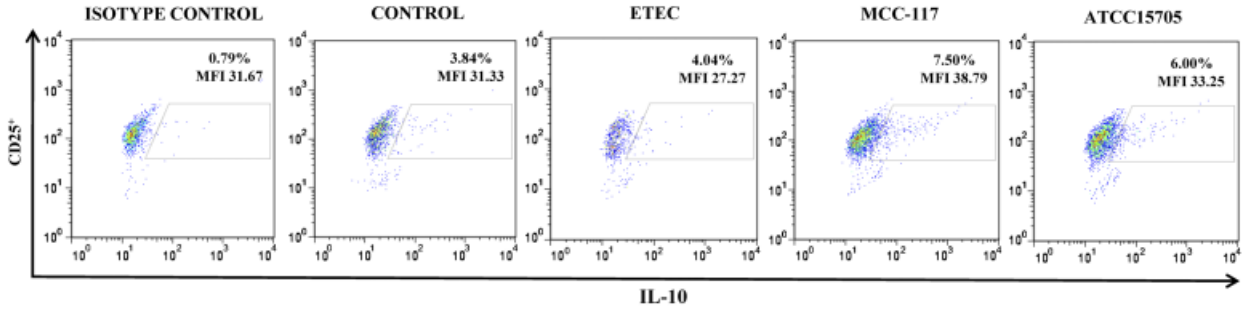

Fig. 6. Expression of IL-10 protein on CD4+CD25high cells after inflammatory stimulation. PIE cells were co-cultured with porcine Peyer's patches immunocompetent cells, pretreated with bifidobacteria (ATCC15705 or MCC-117 strains) for 48 h and then stimulated with ETEC for 12 h. PIE cells without previous treatment (ETEC group) and PIE cells without ETEC challenge (control group) were used as controls. The values of mean fluorescence intensity (MFI) and percentage of positive cells are shown for each group.

critical for T-cell activation because it allows pathogen-specific T cells to overcome the suppressive effect of $CD4^+CD25^+$ Treg cells (Pasare & Medzhitov, 2005). The levels of IL-6 in the $CD4^-CD8^-$ population stimulated with bifidobacteria were lower than those observed in the control, indicating a reduced ability of these APCs to induce the activation of effector T cells. In addition, we have observed previously that the stimulation of PIE cells with lipopolysaccharide or ETEC increased the expression levels of Th1-type cytokines and chemokines (Moue *et al.*, 2008). The release of these mediators, accompanied by the cytokines produced by APCs, would be capable of inducing the stimulation of effector Th1 cells and/or cytotoxic T lymphocytes. Considering that the mechanism of Th1- or cytotoxic T lymphocytes-mediated effector responses implicates the production of IL-2 and IFN-γ, we studied the expression of both cytokines in Peyer's patches $CD4^+CD8^-$ and $CD4^-CD8^+$ cell populations. Our results confirmed that indeed the stimulation with ETEC induced an increase in the levels of IFN-γ in both $CD4^+$ and $CD8^+$ lymphocytes. However, this stimulation was significantly lower in cells treated with *B. breve* MCC-117. The difference in the activation of effector T cells induced by *B. breve* MCC-117 could indicate that the bacterium is able to modify the T effector/T regulatory balance.

The downmodulatory effects of IL-10 on the immune system are many and varied, but include the inhibition of cytokine production by DCs, T cells and macrophages and the inhibition of antigen presentation by DCs. Accordingly, IL-10 has been shown to be important in the control of both T cell- and innate immune cell-mediated intestinal pathology (Coombes & Maloy, 2007). IL-10 is produced by a number of different immune cells, including T cells, B cells, DCs and macrophages. However, IL-10-producing $CD4^+CD25^{high}$ $Foxp3^+$ lymphocytes are the most important cells in regulating the inflammatory response in the intestine (Coombes & Maloy, 2007). Recently, the existence of porcine Tregs, characterized by strong Foxp3 gene expression, IL-10 production and exhibition of the suppressive activity, in the $CD4^+CD25^{high}$ lymphocyte subpopulation, has been reported (Käser *et al.*, 2008). In this work, the levels of IL-10 in porcine Treg cells decreased after a challenge with ETEC. However, in the cells treated with *B. breve* MCC-117, the levels of this cytokine were higher than the control, indicating that the bifidobacteria strain is able to stimulate the Treg cell population.

Although the anti-inflammatory activities of bifidobacteria against ETEC, *Salmonella typhimurium*, lipopolysaccharide or flagellin challenge have been reported previously using IE cell cultures (Riedel *et al.*, 2006; Roselli *et al.*, 2006), this is the first report of the immunoregulatory effect of a bifidobacteria strain on porcine IE cells, which has been selected using the combination of HEK^{pTLR2} immunoassay system and mitogenic assay. We demonstrated that comparative studies on the immunological traits of different bifidobacteria strains using the HEK^{pTLR2} immunoassay system, the mitogenic assay and the PIE-immunocompetent cell co-culture systems could support a rational selection of immunobiotic strains for specific immunomodulatory benefits. These systems could be useful for the evaluation and selection of new immunobiotic bifidobacteria strains, which could be applied in the prevention and treatment of intestinal inflammatory diseases. Furthermore, the comparative study of the anti-inflammatory activity of *B. breve* MCC-117 and *B. adolescentis* ATCC15705, both strains with high mitogenic capacity, demonstrated that the strain able to stimulate HEK^{pTLR2} cells was also the strain with a higher immunoregulatory potential. *Bifidobacterium breve* MCC-117 was more efficient than *B. adolescentis* ATCC15705 in increasing the levels of IL-6 and IL-10 in PIE cells and Treg cells, respectively. Because it has been demonstrated that both effects are mediated via TLR2 signaling (Vinderola

et al., 2005; Zeuthen *et al.*, 2008), the results of this study show that the combination of the HEK^{pTLR2} immunoassay and the evaluation of the mitogenic activity could be an effective primary selection system for candidates of anti-inflammatory immunobiotics.

In conclusion, we found that the combination of a TLR2-mediated cell signaling assay, mitogenic assay and *in vitro* Peyer's patches co-culture system is a useful tool in certain molecular immunoassays for evaluating certain immunobiotic bifidobacteria, and that the different activities of various strains can be accurately detected by these systems. We also found that the previous interaction of immunobiotic bifidobacteria strain with PIE cells is able to significantly modulate the response of immunocompetent cells to challenge with ETEC. The modification induced by bifidobacteria in PIE cells may contribute to intestinal immune homeostasis by ensuring that the contact of PIE cells or APCs with microbial products does not automatically result in the generation of a potentially destructive inflammatory response. In this sense, it would be of great importance to study the changes in signaling pathways in PIE cells produced by the interaction of bifidobacteria with PRRs, and in particular with TLR2. Another interesting point for future investigations is the influence of immunobiotic bifidobacteria on the interaction of PIE cells with porcine DCs, and the subsequent generation of Treg cells. Both the influence of bifidobacteria on PIE PRRs-signaling pathways and on PIE–DCs interaction is currently under investigation in our laboratory. Moreover, because genomic diversities of bifidobacteria population in the swine gut have been reported (Simpson *et al.*, 2003), selection of the useful bifidobacteria from swine intestine using our *in vitro* systems is an interesting aim for future research. Although our findings should be proven in appropriate experiments *in vivo*, in both human and porcine trials, the results of the present work provide a scientific rationale for the use of *B. breve* MCC-117 to prevent ETEC-induced intestinal chronic inflammation in humans and animals.

Acknowledgments

We sincerely thank Dr H. Inadera (Department of Public Health, Toyama Medical and Pharmaceutical University, Japan) for the gift of the pGLM-ENH-luci vector and also Dr Yasushi Kawai and Ms Junko Nishimura for his helpful discussion and her bacterial technical support, respectively. This study was supported by a Grant-in-Aid for Scientific Research (B)(2) (No. 21380164), Challenging Exploratory Research (No. 20658061) and JSPS fellows (No. 2109335) from the Japan Society for the Promotion of Science (JSPS), the Kieikai Research Foundation and Japan Racing Association to H.K. J.V. was supported by JSPS (Postdoctoral Fellowship for Foreign Researchers, Program No. 21-09335).

References

Alvarez S, Villena J, Tohno M, Salva S & Kitazawa H (2009) Modulation of innate immunity by lactic acid bacteria: impact on host response to infections. *Curr Res Immunol* **3**: 87–126.

Bailey M (2009) The mucosal immune system: recent developments and future directions in the pig. *Dev Comp Immunol* **33**: 375–383.

Blumberg RS (2009) Inflammation in the intestinal tract: pathogenesis and treatment. *Digest Dis* **27**: 455–464.

Coombes JL & Maloy KJ (2007) Control of intestinal homeostasis by regulatory T cells and dendritic cells. *Semin Immunol* **19**: 116–126.

Galdeano CM & Perdigón G (2006) The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clin Vaccine Immunol* **13**: 219–226.

He F, Morita H & Ouwehand AC (2006) *Bifidobacteria* and *lactobacilli* exhibited different mitogenic activity on murine splenocytes. *Inter J Probio Prebio* **1**: 77–82.

Hershberg RM & Mayer LF (2000) Antigen processing and presentation by intestinal epithelial cells-polarity and complexity. *Immunol Today* **21**: 123–128.

Hoarau C, Lagaraine C, Martin L, Velge-Roussel F & Lebranchu Y (2006) Supernatant of *Bifidobacterium breve* induces dendritic cell maturation, activation, and survival through a Toll-like receptor 2 pathway. *J Allergy Clin Immun* **117**: 696–702.

Käser T, Gerner W, Hammer SE, Patzl M & Saalmüller A (2008) Phenotypic and functional characterisation of porcine CD4(+)CD25(high) regulatory T cells. *Vet Immunol Immunop* **122**: 153–158.

Kitazawa H, Yamaguchi T & Itoh T (1992) B-cell mitogenic activity of slime products produced from slime-forming, encapsulated *Lactocccus lactis* ssp. *cremoris*. *J Dairy Sci* **75**: 2946–2951.

Kitazawa H, Tohno M, Shimosato T & Saito T (2008) Development of molecular immunoassay system for probiotics via toll-like receptors based on food immunology. *Anim Sci J* **79**: 11–21.

Lavelle EC, Murphy C, O'Neill LA & Creagh EM (2010) The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. *Mucosal Immunol* **3**: 17–28.

López P, Gueimonde M, Margolles A & Suárez A (2010) Distinct *Bifidobacterium* strains drive different immune responses *in vitro*. *Int J Food Microbiol* **138**: 157–165.

Mohamadzadeh M, Olson S, Kalina WV, Ruthel G, Demmin GL, Warfield KL, Bavari S & Klaenhammer TR (2005) *Lactobacilli* activate human dendritic cells that skew T cells toward T helper 1 polarization. *P Natl Acad Sci USA* **102**: 2880–2885.

Moue M, Tohno M, Shimazu T, Kido T, Aso H, Saito T & Kitazawa H (2008) Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. *Biochim Biophys Acta* **1780**: 134–144.

Pasare C & Medzhitov R (2005) Toll-like receptors: linking innate and adaptive immunity. *Adv Exp Med Biol* **560**: 11–18.

Rescigno M, Lopatin U & Chieppa M (2008) Interactions among dendritic cells, macrophages, and epithelial cells in the gut: implications for immune tolerance. *Curr Opin Immunol* **20**: 669–675.

Riedel CU, Foata F, Philippe D, Adolfsson O, Eikmanns BJ & Blum S (2006) Anti-inflammatory effects of bifidobacteria by inhibition of LPS-induced NF-kappaB activation. *World J Gastroentero* **12**: 3729–3735.

Rimoldi M, Chieppa M, Vulcano M, Allavena P & Rescigno M (2004) Intestinal epithelial cells control dendritic cell function. *Ann NY Acad Sci* **1029**: 66–74.

Rimoldi M, Chieppa M, Salucci V, Avogadri F, Sonzogni A, Sampietro GM, Nespoli A, Viale G, Allavena P & Rescigno M (2005) Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* **6**: 507–514.

Roselli M, Finamore A, Britti MS & Mengheri E (2006) Probiotic bacteria *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic *Escherichia coli* K88. *Brit J Nutr* **95**: 1177–1184.

Salva S, Villena J & Alvarez S (2010) Differential immunomodulatory activity of *Lactobacillus casei* strains isolated from goat milk: impact on intestinal and respiratory infections. *Int J Food Microbiol* **141**: 82–89.

Shimosato T, Kitazawa H, Katoh S, Tohno M, Iliev ID, Nagasawa C, Kimura T, Kawai Y & Saito T (2005a) Augmentation of TH-1 type response by immunoactive AT oligonucleotide from lactic acid bacteria via Toll-like receptor 9 signaling. *Biochem Bioph Res Co* **28**: 782–787.

Shimosato T, Tohno M, Kitazawa H, Katoh S, Watanabe K, Kawai Y, Aso H, Yamaguchi T & Saito T (2005b) Toll-like receptor 9 is expressed on follicle-associated epithelia containing M cells in swine Peyer's patches. *Immunol Lett* **15**: 83–89.

Simpson PJ, Stanton C, Fitzgerald GF & Ross RP (2003) Genomic diversity and relatedness of bifidobacteria isolated from a porcine cecum. *J Bacteriol* **185**: 2571–2581.

Stadnyk AW (1994) Cytokine production by epithelial cells. *FASEB J* **8**: 1041–1047.

Takeda K & Akira S (2005) Toll-like receptors in innate immunity. *Int Immunol* **17**: 1–14.

Tohno M, Shimosato T, Kitazawa H *et al.* (2005) Toll-like receptor 2 is expressed on the intestinal M cells in swine. *Biochem Bioph Res Co* **330**: 547–554.

Tohno M, Shimosato T, Moue M, Aso H, Watanabe K, Kawai Y, Yamaguchi T, Saito T & Kitazawa H (2006) Toll-like receptor 2 and 9 are expressed and functional in gut-associated lymphoid tissues of presuckling newborn swine. *Vet Res* **37**: 791–812.

Tohno M, Shimazu T, Aso H, Kawai Y, Saito T & Kitazawa H (2007a) Molecular cloning and functional characterization of porcine MyD88 essential for TLR signaling. *Cell Mol Immunol* **4**: 369–376.

Tohno M, Shimosato T, Kawai Y, Aso H, Ikegami S, Takemoto N, Saito T & Kitazawa H (2007b) Advanced molecular immunoassay system for immunobiotic lactic acid bacteria using a transfectant of Toll-like receptor 2. *Anim Sci J* **78**: 195–205.

Villena J, Racedo S, Agüero G, Bru E, Medina M & Alvarez S (2005) *Lactobacillus casei* improves resistance to pneumococcal respiratory infection in malnourished mice. *J Nutr* **135**: 1462–1469.

Vinderola CG, Matar C & Perdigón G (2005) Role of the epithelial cells in the immune effects mediated by gram-positive probiotic bacteria. Involvement of Toll-like receptors. *Clin Diagn Lab Immun* **12**: 1075–1084.

Westendorf AM, Fleissner D, Hansen W & Buer J (2010) T cells, dendritic cells and epithelial cells in intestinal homeostasis. *Int J Med Microbiol* **300**: 11–18.

Zeuthen LH, Fink LN & Frøkiaer H (2008) Toll-like receptor 2 and nucleotide-binding oligomerization domain-2 play divergent roles in the recognition of gut-derived *lactobacilli* and *bifidobacteria* in dendritic cells. *Immunology* **124**: 489–502.