

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

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Probiotic bacteria may provide protection against intestinal damage induced by pathogens, but the underlying mechanisms are still largely unknown. We investigated whether *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG (LGG) protected intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic *Escherichia coli* (ETEC) K88, by inhibiting pathogen attachment to the cells, which is the first step of ETEC pathogenicity, and regulating neutrophil recruitment, a crucial component of inflammation. A partial reduction of ETEC adhesion was exerted by probiotics and their culture supernatant fractions either undigested or digested with proteases. ETEC viability was unaffected by the presence of *B. animalis*, LGG or their supernatant fractions in the culture medium, indicating an absence of probiotic bactericidal activity. Probiotics and their supernatant fractions, either undigested or digested with proteases, strongly inhibited the neutrophil transmigration caused by ETEC. Both *B. animalis* and LGG counteracted the pathogen-induced up regulation of IL-8, growth-related oncogene- α and epithelial neutrophil-activating peptide-78 gene expression, which are chemokines essential for neutrophil migration. Moreover, the probiotics prevented the ETEC-induced increased expression of IL-1 β and TNF- α and decrease of transforming growth factor- α , which are regulators of chemokine expression. These results indicate that *B. animalis* MB5 and LGG protect intestinal cells from the inflammation-associated response caused by ETEC K88 by partly reducing pathogen adhesion and by counteracting neutrophil migration, probably through the regulation of chemokine and cytokine expression.

Probiotics: Intestinal cells: Inflammation-associated response: Neutrophil migration: Adhesion

There is a growing interest in the use of food supplements containing probiotic bacteria for their suggested positive influence on human and animal health (Majamaa & Isolauri, 1997; Gorbach, 2000; Guarner & Malagelada, 2003; Mercenier *et al.* 2003). Probiotics are normal inhabitants of microflora that have been defined as non-pathogenic bacteria that, upon ingestion in certain amounts, exert beneficial effects for the host (Ouweland *et al.* 2002). Despite increasing evidence of the beneficial effects of probiotics, there is still a need to extend the investigation to other potential probiotic strains, to better define their benefits and to clarify the mechanisms of their activities, that up to now are still largely unknown (Abbott, 2004).

The health benefits of probiotics that are supported by adequate clinical studies and promising experimental data on animals include the prevention of diarrhoea and intestinal inflammatory diseases (Hampson, 1994; Dotan & Rachmilewitz, 2005; Jenkins *et al.* 2005). Considering that several lactobacilli and bifidobacteria have been proven to maintain antibiotic susceptibility (Zhou *et al.* 2005), probiotic feeding may represent an important antibiotic alternative therapy for animals and human subjects to prevent the enteropathogen

infections. This is particularly important in the case of piglets, where the large use of antibiotics to prevent their frequent intestinal infections may have serious consequences on human health, due to the possible presence of antibiotic-resistant bacteria in pig food products and in animal waste-contaminated environments (Smith *et al.* 2002).

Several probiotics have been shown to inhibit pathogen adhesion (Bernet *et al.* 1994; Jin *et al.* 2000; Forestier *et al.* 2001). This could be the mechanism of probiotic protection against enterotoxigenic *Escherichia coli* (ETEC) infection, which should attach to mucosal surfaces to release toxins responsible for the development of diarrhoea and inflammation (Hampson, 1994). However, there is increasing evidence that probiotics may act, not only by competing for pathogen adhesion, but also through diverse mechanisms, including maintenance of mucosal barrier integrity and modulation of mucosal immune response (Ouweland *et al.* 2002; Teitelbaum & Walker, 2002; Vaughan *et al.* 2002; O'Sullivan *et al.* 2005). Enteric pathogens activate the inflammatory cascade by a complex of not yet completely understood signals (Neish, 2002; Berkes *et al.* 2003).

Abbreviations: CFU, colony-forming units; D-MEM, Dulbecco's modified minimum essential medium; ENA, epithelial neutrophil-activating peptide; ETEC, enterotoxigenic *Escherichia coli*; GRO, growth-related oncogene; HBSS, Hank's balanced salt solution; LB, Luria-Bertani; LGG, *Lactobacillus rhamnosus* GG; MPO, myeloperoxidase; TGF, transforming growth factor.

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The final response is the recruitment of neutrophils and other inflammatory cells induced by chemokines, such as IL-8, growth-related oncogene (GRO)- α and epithelial neutrophil-activating peptide (ENA)-78 (Savkovic *et al.* 1996; Jaye & Parkos, 2000), which in turn are regulated by several cytokines (Baggiolini *et al.* 1995; Schroder, 2000; Rudack *et al.* 2003). Although neutrophils represent the primary line of host defence against micro-organisms, a massive and prolonged infiltration of neutrophils may perpetuate inflammation and ultimately lead to cell damage, epithelial barrier dysfunction and pathophysiology of diarrhoea (Elliott & Wallace, 1998; Gewirtz *et al.* 2002). Thus, the prevention of excessive neutrophil recruitment may represent a tool to counteract the development of pathogen-induced inflammatory reactions. A reduction of inflammatory cell infiltration by certain lactobacilli and bifidobacteria in chemically or pathogen-induced intestinal inflammation has been reported (Hidemura *et al.* 2003; Michail & Abernathy, 2003; Peran *et al.* 2005). However, it is not clear whether the regulation of inflammatory cell migration is a common effect of probiotics. Indeed, not all probiotic species exert the same anti-inflammatory activity in the intestine (Shibolet *et al.* 2002; Ibnou-Zekri *et al.* 2003).

In the present study we have used an *in vitro* model of a human intestinal cell line, the Caco-2 cell, to investigate whether *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG (LGG) were able to protect the cells against the inflammation-associated response caused by ETEC K88. We have investigated whether the potential protection could be only due to impediment of pathogen attachment to the cells or, more specifically, to a regulation of neutrophil recruitment. Caco-2 cells are well characterised enterocyte-like cells, able to differentiate into mature enterocytes (Neutra & Louvard, 1989), and have been extensively used to study probiotic effects (Lehto & Salminen, 1997; Lievin-Le Moal *et al.* 2002; Resta-Lenert & Barrett, 2003). The strain *B. animalis* MB5 has not been previously investigated for its potential activity against infection, but in our previous study it was able to counteract Zn deficiency-induced intestinal damage (Mengheri *et al.* 1999). LGG was chosen for its documented protection against intestinal disease (Majamaa *et al.* 1995; Gorbach, 2000; Marteau *et al.* 2001; Dieleman *et al.* 2003). ETEC K88 was used as an enterotoxigenic bacterium able to adhere to Caco-2 cells and to induce intestinal damage, as shown in our previous study (Roselli *et al.* 2003).

Materials and methods

Epithelial cell culture

The human intestinal Caco-2 cells were grown in Dulbecco's modified minimum essential medium (D-MEM; 3.7 g NaHCO₃/l, 4 mM-glutamine, 100 ml heat-inactivated fetal calf serum/l, 10 ml non-essential amino acids/l, 100 kU penicillin/l and 100 mg streptomycin/l). All cell-culture reagents were from Biochrom KG (Milan, Italy). The cells were maintained at 37°C in an atmosphere of 5% CO₂-95% air at 90% relative humidity. After confluency, cells were left for 17–21 d to allow differentiation, as previously reported (Roselli *et al.* 2003).

Bacterial strains

ETEC strain K88 (provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Reggio Emilia, Italy) was grown in Luria-Bertani (LB) medium, as previously reported (Roselli *et al.* 2003). After overnight incubation at 37°C with shaking, subcultures of bacteria grown until mid-log phase were centrifuged at 4000 g for 10 min at 4°C and washed in PBS. Bacterial cells were then re-suspended in antibiotic-free D-MEM for experiments on adhesion, ETEC survival and chemokine and cytokine expression, or in Hank's balanced salt solution (HBSS; 137 mM-NaCl, 5.36 mM-KCl, 1.67 mM-CaCl₂, 1 mM-MgCl₂, 1.03 mM-MgSO₄, 0.44 mM-KH₂PO₄, 0.34 mM-Na₂HPO₄, 5.6 mM-glucose) for neutrophil transmigration experiments.

B. animalis strain MB5 (ATCC25527; provided by Bruno Biavati, University of Bologna, Bologna, Italy) and *L. rhamnosus* strain GG (ATCC53103) were grown in de Man Rogosa Sharpe medium (Difco, Milan, Italy). After overnight incubation at 37°C in anaerobic conditions, subcultures of probiotics were grown until mid-log phase and prepared as described for ETEC.

Bacterial concentrations of both ETEC and probiotics were estimated in preliminary experiments by densitometry followed by colony-forming units (CFU) counts after agar plating of bacterial serial dilutions.

The viability of ETEC, *B. animalis* and LGG grown in D-MEM for the time of the different experiments (1.5–2 h) did not differ from that of bacteria grown in LB or de Man Rogosa Sharpe media, as tested by agar plating in preliminary experiments.

Preparation of spent probiotic supernatant fractions and proteolytic digestion

Spent culture supernatant fractions were prepared from overnight cultures of *B. animalis* and LGG after centrifugation at 4000 g for 10 min at 4°C followed by filtration of the supernatant fractions through a 0.22 μ m pore-size filter, to remove any remaining bacteria. Samples of supernatant fractions were incubated with trypsin (200 μ g/ml) and proteinase K (100 μ g/ml) at 37°C for 12 h, and then with 1 mM-phenyl methyl sulfonyl fluoride (Sigma, Milan, Italy) to stop the reaction. Enzymes were from Sigma. The efficacy of digestion was checked on SDS-polyacrylamide gel electrophoresis.

Enterotoxigenic Escherichia coli K88 growth in the presence of probiotics or their culture media

To test whether probiotics or their secreted factors could exert a bactericidal activity, ETEC (10⁸ CFU/ml) was grown in 1 ml D-MEM with or without different amounts of *B. animalis* or LGG (10⁹ and 10¹⁰ CFU/ml), or their equivalent spent culture supernatant fractions, at 37°C in anaerobiosis for 2 h. This assay was performed in twenty-four-well plates to reproduce the static conditions used in all experiments. ETEC survival was verified by agar plating.

Bacterial adhesion

Caco-2 cells (10⁶ cells/well) were differentiated in twenty-four-well plates. To test the protective effect of probiotics

against ETEC adhesion, cells were treated with 1 ml D-MEM containing ETEC alone (10^8 CFU) or ETEC together with different amounts of *B. animalis* or LGG (10^9 and 10^{10} CFU). The concentration of probiotics with respect to ETEC was chosen according to previous studies (Bernet *et al.* 1994; Forestier *et al.* 2001). To determine if soluble factors released by probiotics could inhibit ETEC adhesion, samples of *B. animalis* and LGG culture supernatant fractions equivalent to 10^9 and 10^{10} CFU were added to the cells in 1 ml D-MEM containing ETEC (10^8 CFU). To test if the effect of the spent supernatant fraction was due to a proteinaceous factor, samples of supernatant fractions equivalent to 10^9 and 10^{10} CFU digested with proteases were added to the cells in 1 ml D-MEM containing ETEC (10^8 CFU). Since previous studies reported that addition of bacterial medium to D-MEM can reduce the pH, and that a pH value below 5.8 affects Caco-2 cells (Lehto & Salminen, 1997), supernatant fractions in D-MEM were checked for their pH and those equivalent to 10^{10} CFU were neutralised with NaOH. After incubation of Caco-2 cells with ETEC, probiotics or their spent supernatant fractions at 37°C for 1.5 h, non-adhered bacteria were removed by five washes with HBSS and monolayers lysed with Triton-X-100 (10 ml/l). Viable ETEC K88 were quantified by plating on LB agar appropriate serial dilutions of lysates. Preliminary experiments have shown that *B. animalis* MB5 and LGG were not able to form colonies after overnight incubation on LB agar at 37°C in aerobic conditions. In preliminary experiments supernatant fractions were also evaluated for any potential negative effect on membrane integrity. The transepithelial electrical resistance was measured according to Roselli *et al.* (2003), and was unaffected. Adhesion of probiotics was also tested by treating the cells with 1 ml D-MEM containing *B. animalis* or LGG (10^9 and 10^{10} CFU). Viable probiotics were quantified by de Man Rogosa Sharpe agar plating.

Isolation of neutrophils

Neutrophils were freshly isolated from blood of healthy volunteers. Heparin-anticoagulated blood was diluted with ice-cold PBS, washed, layered on top of Ficoll-paque plus (Amersham Bioscience, Milan, Italy) and centrifuged for 40 min at 600 g. After two washes, the pellet was diluted in ice-cold solution containing 0.155 M-NH₄Cl, 10 mM-KHCO₃ and 0.1 mM-EDTA, and kept on ice to lyse erythrocytes. After centrifugation, neutrophils were washed, re-suspended in HBSS and used within 1 h from isolation. Neutrophils were typically 95% pure with 98% viability as assessed by trypan blue exclusion.

Neutrophil transmigration

Caco-2 cells (1.5×10^5 cells/filter) were differentiated as an inverted monolayer in Transwell filters (polyethylene terephthalate filter inserts for cell culture of 6.5 mm diameter, 3.0 µm pore diameter; Becton Dickinson, Milan, Italy), to allow the physiological neutrophil migration from basolateral to apical compartment, according to Parkos *et al.* (1991). The filters were inserted into twenty-four-well plates. The integrity of inverted monolayers was checked by transepithelial electrical resistance. The monolayers were kept in serum-free medium

overnight before the transmigration experiment. Neutrophils (10^6 cells/well) were added to the basolateral compartment (upper reservoir) of the Transwell filters. Bacterial cells were then added to the apical compartment (lower reservoir) in 0.5 ml HBSS containing either ETEC (10^7 CFU/ml), *B. animalis* and LGG (10^8 CFU/ml) separately, or ETEC together with *B. animalis* or LGG. To test the effect of killed probiotics, *B. animalis* and LGG (10^8 CFU/ml) re-suspended in HBSS were heat-treated for 30 min at 95°C, and then added to the cells simultaneously with ETEC. Killing of bacteria was confirmed by agar plating. Probiotic culture supernatant fractions equivalent to the bacterial cells present in 0.5 ml HBSS (5×10^7 CFU), either undigested or digested with proteases, were added simultaneously with ETEC. The neutrophils were allowed to transmigrate for 2 h at 37°C. Neutrophil viability was always checked at the end of the experiments by trypan blue exclusion. The concentration of ETEC was chosen on the basis of preliminary experiments showing that ETEC did not affect the transepithelial electrical resistance during the 2 h of the experiments and thus did not allow an indiscriminate neutrophil passage. The bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (1 µM; Sigma), which acts as a strong chemoattractant (Schroder, 2000), was used as a positive control.

Chemotaxis assay

All transmigrated neutrophils (within the monolayer + apical compartment), were measured by myeloperoxidase (MPO) activity, which is a specific marker of neutrophil identification, as previously described (Parkos *et al.* 1991). Briefly, samples from the apical compartment were solubilised in HBSS containing Triton X-100 and citrate buffer (pH 4.2), to a final concentration of 0.5 ml/l and 0.1 mM, respectively. Monolayers were washed twice with HBSS and the filters gently scraped and solubilised in the same lysis buffer. MPO activity was assayed by reading the optical density at 405 nm, after the addition of an equal volume of 0.1 mM-citrate buffer (pH 4.2) containing 1 mM-2,2'-azino-di-(3-ethyl)dithiazoline sulfonic acid (Sigma) and 10 mM-H₂O₂ (Sigma). After appropriate colour development, the reaction was stopped by SDS (5 g/l). The number of transmigrated neutrophils was calculated from a standard curve established from known concentrations of the same neutrophil preparation used in each experiment. The standard curve was linear in the range of 0.5–50 × 10⁴ cells. The measured MPO activity was negligible in lysates of Caco-2 monolayers unexposed to neutrophils and accurately reflected neutrophil-associated MPO, since cell-free supernatant fractions of apical compartments contained less than 3% of total MPO activity.

Cytokine mRNA

Caco-2 cells (4×10^6 cells/well) were differentiated in six-well plates. Cells were treated with 4 ml D-MEM containing either ETEC (10^8 CFU/ml), *B. animalis* and LGG (10^9 CFU/ml) separately, or ETEC together with *B. animalis* or LGG, for 2 h. Preliminary experiments indicated that 2 h of bacterial treatments induced the most evident changes in

cytokine mRNA levels. Total RNA was extracted with TRIZOL reagent (Invitrogen, Milan, Italy) and 1 μ g RNA was subjected to RT-PCR assay, as previously described (Finamore *et al.* 2003). The abundances of mRNA encoding transforming growth factor (TGF)- β 1, TNF- α , IL-1 β , IL-8, GRO- α and ENA-78 were analysed. Each cytokine was co-amplified with glyceraldehyde-3-phosphate-dehydrogenase as an internal control. The sequences of TGF- β 1, IL-8, TNF- α , GRO- α and glyceraldehyde-3-phosphate-dehydrogenase primers are reported in a previous study (Roselli *et al.* 2003). The sequences of the other primers were the following: IL-1 β sense 5'-GAT CAT CTG TCT CTG AAT CA-3' and antisense 5'-TCC AGA TTA TGT AAT GCA GC-3' (expected fragment size 300 bp); ENA-78 sense 5'-TTC GCC ATA GGC CCA CA-3' and antisense 5'-CAA ATT TCC TTC CCG TTC TTC A-3' (expected fragment size 177 bp). The primers were provided by MWG Biotech (Firenze, Italy). The PCR products were analysed on 2% agarose gels and the relative intensity of the bands was measured by Scion image software (Scion Corporation, Frederick, MD, USA). The cytokine:glyceraldehyde-3-phosphate-dehydrogenase mRNA intensities ratio was used to evaluate the relative levels of expressions.

Statistical analysis

The data are expressed as mean values and standard deviations. The significance of the differences was evaluated by one-way ANOVA followed by Fisher's test. Differences with $P < 0.05$ were considered significant. Regression analysis was used to evaluate the linear relationship between the migration of neutrophils and the expression of chemokines or cytokines. Multiple regression analysis among chemokines plus cytokines and neutrophil migration was used. All statistical analyses were performed with SAS release 8.1 statistical software (SAS Institute, Cary, NC, USA).

Results

Reduction of enterotoxigenic *Escherichia coli* K88 adhesion

As we have previously shown (Roselli *et al.* 2003), ETEC was able to adhere to Caco-2 cells after 1.5 h of infection (Fig. 1(A)). Also *B. animalis* and LGG adhered to the cells, and more efficiently by increasing the concentration of bacteria (up to 6×10^7 CFU/ml; data not shown). Treatment with *B. animalis* or LGG induced a reduction of ETEC K88 adhesion (Fig. 1(A); $P < 0.05$). The inhibition of pathogen attachment was greater by increasing *B. animalis* (10^{10} CFU/ml; $P < 0.001$), but not LGG concentration. When spent supernatant fractions of probiotic cultures were added to the cells to test the possibility of an inhibitory activity of a secreted factor, a strong reduction of ETEC adhesion was observed with the culture medium of *B. animalis* and LGG equivalent to 10^9 and 10^{10} CFU/ml respectively (Fig. 1(B); $P < 0.001$). The supernatant fraction of LGG equivalent to 10^9 CFU/ml was ineffective in reducing the attachment of ETEC to the cells (data not shown). Proteolytic digestion of culture supernatant fractions did not affect the ability to reduce the ETEC adhesion (Fig. 1(B)).

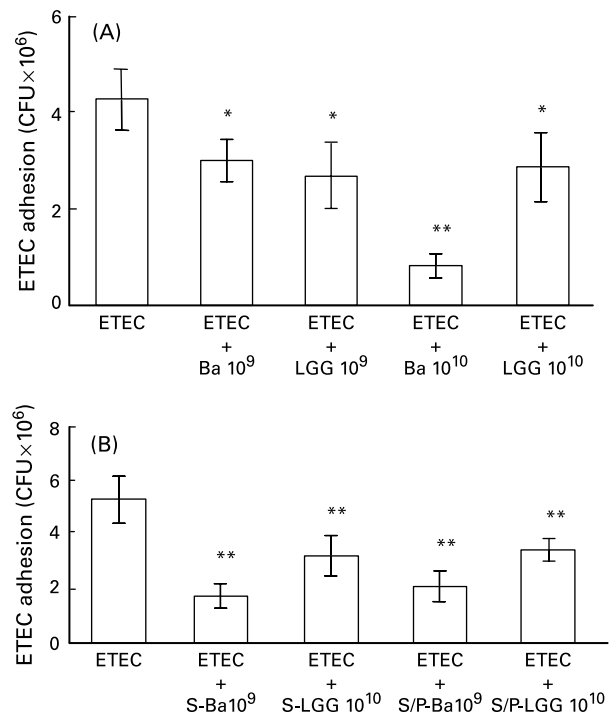


Fig. 1. Reduction of enterotoxigenic *Escherichia coli* (ETEC) K88 adhesion by *Bifidobacterium animalis* MB5 (Ba) and *Lactobacillus rhamnosus* GG (LGG). (A) Effect of probiotics; (B) effect of spent supernatant fractions either undigested (S-Ba and S-LGG) or digested with proteases (S/P-Ba and S/P-LGG). Caco-2 cells (10^6 cells/well) differentiated in twenty-four-well plates were treated with ETEC alone (10^8 colony-forming units (CFU)/ml), or ETEC together with Ba (10^9 and 10^{10} CFU/ml), LGG (10^9 and 10^{10} CFU/ml) or their culture supernatant fractions (equivalent to 10^9 Ba and 10^{10} LGG) simultaneously with ETEC, for 1.5 h. Adhered ETEC was determined by agar plating-appropriate dilutions of cell lysates and reported as CFU. Values are means, with standard deviations represented by vertical bars ($n = 5$). Mean value was significantly different from that of the ETEC group: * $P < 0.05$; ** $P < 0.001$.

Effect of probiotics on enterotoxigenic *Escherichia coli* K88 survival

The viability of ETEC grown alone did not significantly differ from that of ETEC grown in medium containing *B. animalis* or LGG, with both 10^9 and 10^{10} CFU/ml (Fig. 2). Also the different concentrations of spent supernatant fractions of both probiotics did not affect ETEC viability and the results of supernatant fractions equivalent to 10^9 *B. animalis* and 10^{10} LGG, which are those used in the adhesion experiments, are shown in Fig. 2.

Reduction of enterotoxigenic *Escherichia coli*-induced neutrophil transmigration

ETEC induced a strong neutrophil transmigration across the inverted monolayer of Caco-2 cells after 2 h of infection, to a similar extent to that caused by the N-formyl-methionyl-leucyl-phenylalanine chemoattractant (Fig. 3(A); $P < 0.001$). Treatment of uninfected cells with *B. animalis* or LGG induced only a small transmigration ($P < 0.05$). When *B. animalis* or LGG were added to the cells simultaneously with ETEC, a marked reduction of neutrophil migration was observed (Fig. 3(A); $P < 0.001$). Conversely, no reduction

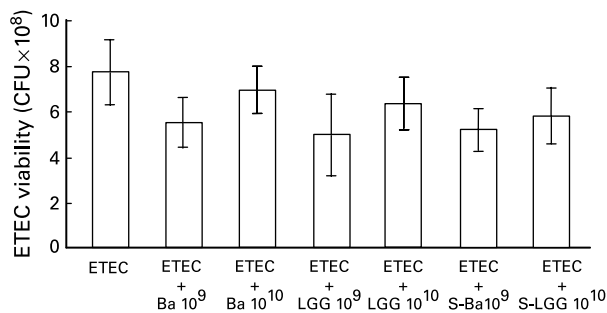


Fig. 2. Effect of *Bifidobacterium animalis* MB5 (Ba) and *Lactobacillus rhamnosus* GG (LGG) on enterotoxigenic *Escherichia coli* (ETEC) K88 viability. ETEC (10^8 colony-forming units (CFU)/ml) was grown in twenty-four-well plates alone or in the presence of probiotics (10^9 and 10^{10} CFU/ml) or their spent supernatant fractions (equivalent to 10^9 Ba and 10^{10} LGG), for 2 h. Viability of ETEC was determined by agar plating-appropriate dilutions of bacterial cultures and reported as CFU. Values are means, with standard deviations represented by vertical bars (n 3).

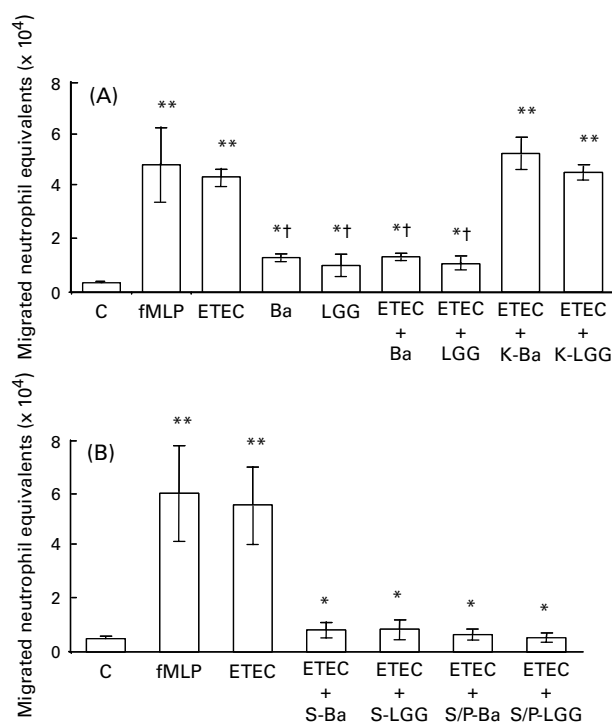


Fig. 3. Effect of *Bifidobacterium animalis* MB5 (Ba) and *Lactobacillus rhamnosus* GG (LGG) on neutrophil migration induced by enterotoxigenic enterotoxigenic *Escherichia coli* (ETEC) K88. (A) Effect of live or killed (K) probiotics; (B) effect of spent supernatant fractions either undigested (S-Ba and S-LGG) or digested with proteases (S/P-Ba and S/P-LGG). Caco-2 cells differentiated as an inverted monolayer on Transwell filters (1.5×10^5 cells/filter) were untreated (control; C) or treated with 0.5-ml Hank's balanced salt solution containing ETEC (10^7 colony-forming units (CFU)/ml), live probiotics (10^8 CFU/ml) separately or simultaneously with ETEC, killed probiotics (equivalent to 5×10^7 CFU) or spent probiotic supernatant fractions (equivalent to 5×10^7 CFU) simultaneously with ETEC. The number of transmigrated neutrophils was quantified by myeloperoxidase assay. N-formyl-methionyl-leucyl-phenylalanine (fMLP; $1 \mu\text{M}$) was used as the positive control. Values are means, with standard deviations represented by vertical bars (n 6). Mean value was significantly different from that of group C: * $P < 0.05$; ** $P < 0.001$. † Mean value was significantly different from that of the ETEC group ($P < 0.001$).

was found when heat-killed probiotics were used (Fig. 3(A)). Spent medium from probiotic cultures was able to reduce the number of transmigrated neutrophils (Fig. 3(B); $P < 0.001$). The decrease of neutrophil migration was maintained after proteolytic digestion of culture supernatant fractions (Fig. 3(B)).

Chemokine and cytokine gene expressions

Infection of Caco-2 cells with ETEC caused a strong up regulation of the chemokines IL-8, GRO- α and ENA-78 (Fig. 4 (A); $P < 0.001$). When the two probiotics were added alone to the cells, a small increase of IL-8, significant only with LGG ($P < 0.05$), and an up regulation of ENA-78 by both *B. animalis* ($P < 0.05$) and LGG ($P < 0.001$) were observed. When Caco-2 cells were treated with probiotics simultaneously with ETEC, the IL-8, GRO- α and ENA-78 expressions were markedly lower than those found after ETEC infection ($P < 0.01$) and did not differ from untreated cells.

An up regulation of the pro-inflammatory cytokines IL-1 β and TNF- α , as well as a down regulation of the anti-inflamma-

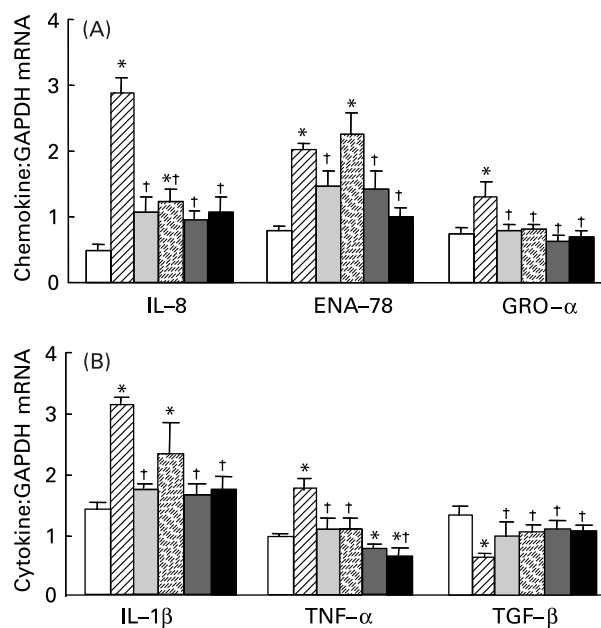


Fig. 4. Effect of *Bifidobacterium animalis* MB5 (Ba) and *Lactobacillus rhamnosus* GG (LGG) on alterations of chemokine and cytokine gene expressions induced by enterotoxigenic *Escherichia coli* (ETEC) K88. (A) Expression of the chemokines IL-8, growth-related oncogene (GRO)- α and epithelial neutrophil-activating peptide (ENA)-78; (B) expression of the cytokines IL-1 β , TNF- α and transforming growth factor (TGF)- β 1. Caco-2 cells (4×10^6 cells/well) differentiated in six-well plates were untreated (control; □) or treated with 4 ml medium containing ETEC (10^8 colony-forming units (CFU)/ml; ▨) and probiotics (10^9 CFU/ml), either separately (Ba ■; LGG ▩) or simultaneously with ETEC (ETEC + Ba ■; ETEC + LGG ▩), for 2 h. Gene expressions were analysed by RT-PCR. The densitometric values of each cytokine mRNA normalised to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA are represented. Values are means, with standard deviations represented by vertical bars (n 5). * Within each chemokine or cytokine, mean value was significantly different from that of the control group ($P < 0.05$). † Within each chemokine or cytokine, mean value was significantly different from that of the ETEC group ($P < 0.05$).

Table 1. Linear regression analysis of association of neutrophil migration with chemokines and cytokines

	<i>R</i>	<i>P</i>
Chemokines and neutrophils*		
IL-8	0.91	<0.001
Epithelial neutrophil-activating peptide-78	0.49	<0.01
Growth-related oncogene- α	0.88	<0.001
Cytokines and neutrophils*		
IL-1 β	0.81	<0.001
TNF- α	0.80	<0.001
Transforming growth factor- β 1	-0.73	<0.001

* Data were grouped by treatment.

tory cytokine TGF- β 1, were induced by ETEC K88 infection (Fig. 4 (B); $P < 0.001$). Treatment of the cells with *B. animalis* or LGG alone did not induce any change on the expression of these cytokines, with the exception of an increase of IL-1 β by LGG ($P < 0.05$). The addition of *B. animalis* or LGG to the cells simultaneously with ETEC was effective in maintaining the expression of these cytokines at the level of untreated cells. The regression analysis between neutrophil migration and chemokines indicates a highly positive association with IL-8 and GRO- α , and a low association with ENA-78. Moreover, neutrophil migration was correlated positively with IL-1 β and TNF- α , and negatively with TGF- β 1 (Table 1). Multiple regression analysis among chemokines plus cytokines and neutrophil migration indicated that the main effect was the IL-8 expression (R 0.91; $P < 0.001$).

Discussion

More information on the beneficial effects of different probiotic strains and scientific evidence behind their activities are still needed for a wider use of probiotics as a beneficial food supplement to prevent intestinal inflammation. We addressed whether *B. animalis* MB5 and LGG protected Caco-2 cells from the inflammation-associated response triggered by ETEC K88, by inhibiting the pathogen adhesion and regulating the recruitment of neutrophils, which represent a crucial component of inflammation, using Caco-2 cells as a model epithelium.

The first step in the pathogenesis of ETEC is the attachment to intestinal cells that allows the enterotoxin release (Jin & Zhao, 2000). Thus, we first investigated whether the two strains of probiotics were able to inhibit the adhesion of ETEC to the cells. The present study shows that treatment of Caco-2 cells with *B. animalis* and LGG induced a considerable protection against the pathogen adhesion, whose efficacy improved with increased concentration of *B. animalis*.

The present study also shows that the supernatant fractions of overnight cultures of *B. animalis* and LGG counteracted the ETEC adhesion, suggesting that one or more soluble factors released by probiotics were able to induce this effect. The inhibition of ETEC adhesion was not due to a bactericidal protein, since the viability of ETEC was unaffected when it was grown in culture medium containing *B. animalis* LGG or their culture supernatant fractions. Moreover, the released factor was not a protein, since the supernatant fractions digested with proteases maintained the ability to inhibit ETEC

adhesion. Previous data have indicated that the acidic pH of the culture medium linked to organic acid production was the determinant factor for pathogen adhesion inhibition by probiotics (Lehto & Salminen, 1997). However, we can exclude the possibility that the reduction of ETEC adhesion was due to a pH effect, since culture supernatant fractions were at neutral pH values. All together, the present results suggest that the impairment of the ETEC adhesion by *B. animalis* and LGG may be achieved by either competition for the binding sites to the cells and/or secretion of an active, non-proteinaceous factor, in accordance with previous findings (Granato *et al.* 1999; Jin *et al.* 2000).

Although strongly reduced, the ETEC adhesion was not completely inhibited by the two probiotics; thus a certain number of the pathogen did attach to the cells and could trigger the inflammatory reactions. When the two probiotics were added together with ETEC to the cells, both *B. animalis* and LGG were able to strongly reduce the migration of inflammatory cells induced by the pathogen. We have also found that while heat-killed bacteria did not maintain the ability to inhibit the ETEC-induced neutrophil migration, spent culture media of both *B. animalis* and LGG were effective in reducing this migration. As seen for the adhesion, the inhibitory activity was maintained after proteolytic cleavage of the supernatant fractions. Thus, these results suggest that either live probiotics and/or a non-proteinaceous factor released by probiotics were able to interfere with inflammatory cell recruitment. Additional experiments are needed to determine the precise nature of the soluble factor released by probiotics and its protective role.

The neutrophil migration caused by ETEC was probably induced by chemokines, which are essential for their recruitment (Jaye & Parkos, 2000), since a strong increase of IL-8, GRO- α and ENA-78 occurred after infection of the cells. The two probiotics were able to maintain the expression of chemokines at the level of untreated cells. The strong relationship between the number of migrated neutrophils and the expression of IL-8 and GRO- α are in favour of a link between inhibition of inflammatory cell recruitment and chemokine expression. Interestingly, treatment of uninfected cells with *B. animalis* or LGG induced only a low neutrophil migration that was associated with a small increase of IL-8 and, unexpectedly, with a strong up regulation of ENA-78 by LGG. This last chemokine is expressed by different cell types after stimulation with pro-inflammatory mediators, but its role in inflammation is unclear (Keates *et al.* 1997; Rollins, 1997; Szekanecz *et al.* 1998). While IL-8 and GRO- α are the most potent neutrophil chemotactic proteins (Baggiolini *et al.* 1995; Schroder, 2000), ENA-78 has been found to be secreted later and to a lower extent than IL-8, and up to 100-fold less potent than IL-8 in attracting neutrophils to inflammatory sites (Walz *et al.* 1993). Thus, an up regulation of ENA-78 does not necessarily lead to an activation of neutrophils, and this probably happened in our experimental conditions. Despite the fact that the increase in ENA-78 remains to be explained, overall the present results indicate that the two probiotics might differently regulate chemokine expression, depending on whether they were added alone or together with ETEC to the cells.

The expression of chemokines may be regulated by several cytokines. Among them, TNF- α and IL-1 β can up

regulate IL-8, GRO- α and ENA-78 (Schroder, 2000; Rudack *et al.* 2003; Jarai *et al.* 2004). Conversely, TGF- β can prevent the production of TNF- α and down regulate IL-8 production (Lugering *et al.* 1998). In our conditions, ETEC caused an up regulation of IL-1 β and TNF- α , and a down regulation of TGF- β 1. These changes might have led to the increase of chemokine expression. On the other hand, *B. animalis* and LGG were able to counteract the ETEC-induced alterations of cytokine expression, and consequently to block the chemokine dysregulation. These results are in agreement with previous findings that probiotics may suppress the inflammatory response to an infectious stimulus through regulation of pro-inflammatory cytokine expression (Gill, 2003; Otte *et al.* 2004). Interestingly, an increase of IL-1 β was induced when LGG was added to uninfected cells. The increase of IL-1 β is not completely surprising, since there is evidence that some strains of lactobacilli may up regulate pro-inflammatory cytokines (Haller *et al.* 2000; Jijon *et al.* 2004). The fact that LGG, but not *B. animalis*, stimulates IL-1 β is in favour of the idea that individual strains of probiotics may have a different impact on the immune system, as previously indicated (Ibnou-Zekri *et al.* 2003; Jijon *et al.* 2004). In addition, the results of the absence of IL-1 β increase when LGG and *B. animalis* were added simultaneously with ETEC to the cells further confirm that the immunomodulatory activity of the two probiotics may be different in the presence or absence of the pathogenic stimulus, as also suggested earlier for the chemokine regulation. This finding is important considering that the co-existence of probiotics with pathogens represents the physiological intestinal condition of man and animals.

It should be considered that in the present study only the gene expression of cytokines and chemokines was evaluated, which does not necessarily reflect the synthesis of the proteins, and thus we cannot exclude the possibility of a different protein modulation by probiotics. For example, TGF- β needs activation to be effective. However, it has been shown that TGF- β 1 activation occurred after pathogen infection, and we can presume that this happened in our experimental model of bacterial treated cells (Nagineni *et al.* 2002). In addition, a good correspondence between cytokine and chemokine transcript and protein level has been reported by several studies (Berin *et al.* 2002; Haller *et al.* 2002; Kim *et al.* 2005).

In conclusion, we report a novel ability of both *B. animalis* MB5 and LGG to protect intestinal cells against the inflammation-associated response caused by ETEC K88. This protection was achieved by partly reducing ETEC adhesion and by counteracting the neutrophil migration probably through a reduction of chemokine increase, mediated by regulation of pro- and anti-inflammatory cytokines. Although our findings should be proven *in vivo*, the present results may provide a scientific rationale for the use of *B. animalis* MB5 and LGG to prevent or alleviate ETEC-induced intestinal disorders.

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