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 ETEC induced expression of IL-1β, 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 increase of IL-8, growth-related oncogene-α and epithelial neutrophil-activating peptide-78 gene expression, which are chemokines essential for neutrophil migration. Furthermore, the probiotics prevented the ETEC-induced increase of IL-1β 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 (Ouwehand 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 (Ouwehand 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; Ibnou-Zekri 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; Ibnou-Zekri et al. 2003). 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 (Shibol et al. 2002; Ibou-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; Neutra & Louvard, 1989), and have been extensively 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).

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 μm pore-size filter, to remove any remaining bacteria. Samples of supernatant fractions were incubated with trypsin (200 μg/ml) and proteinase K (100 μg/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 (10⁶ CFU/ml) was grown in 1 ml D-MEM with or without different amounts of B. animalis or LGG (10⁶ 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 (10⁸ CFU) or ETEC together with different amounts of *B. animalis* or LGG (10⁹ and 10¹⁰ 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 10⁹ and 10¹⁰ CFU were added to the cells in 1 ml D-MEM containing ETEC (10⁸ CFU). To test if the effect of the spent supernatant fraction was due to a proteinaceous factor, samples of supernatant fractions equivalent to 10⁹ and 10¹⁰ CFU digested with proteases were added to the cells in 1 ml D-MEM containing ETEC (10⁸ 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 10¹⁰ 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 (10⁹ and 10¹⁰ 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 × 10⁴ 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 (10⁶ 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 (10⁵ CFU/ml), *B. animalis* and LGG (10⁸ CFU/ml) separately, or ETEC together with *B. animalis* or LGG. To test the effect of killed probiotics, *B. animalis* and LGG (10⁶ 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 × 10⁵ 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 (Schröder, 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 × 10⁵ 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 × 10⁶ cells/well) were differentiated in six-well plates. Cells were treated with 4 ml D-MEM containing either ETEC (10⁸ CFU/ml), *B. animalis* and LGG (10⁸ 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 μg RNA was subjected to RT-PCR assay, as previously described (Finamore et al. 2003). The abundances of mRNA encoding transforming growth factor (TGF)-β1, TNF-α, IL-1β, 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-β1, 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-1β 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 $6 \times 10^7$ 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)).

**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
Probiotic protection against Escherichia coli

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-\( \alpha \) 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-\( \alpha \) and ENA-78 expressions were markedly lower than those found after ETEC infection (\( P<0.001 \)) and did not differ from untreated cells.

An up regulation of the pro-inflammatory cytokines IL-1\( \beta \) and TNF-\( \alpha \), as well as a down regulation of the anti-inflamma-

Fig. 3. Effect of \( B. \) animalis MB5 (Ba) and \( L. \) rhamnosus GG (LGG) on neutrophil migration induced by enterotoxigenic \( E. \) coli (ETEC) K88 viability. ETEC (\( 10^6 \) colony-forming units (CFU/ml) was grown in twenty-four-well plates alone or in the presence of probiotics (\( 10^7 \) and \( 10^9 \) CFU/ml) or their spent supernatant fractions (equivalent to \( 5 \times 10^9 \) 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. 4. Effect of \( B. \) animalis MB5 (Ba) and \( L. \) rhamnosus GG (LGG) on alterations of chemokine and cytokine gene expressions induced by enterotoxigenic \( E. \) coli (ETEC) K88. (A) Expression of the chemokines IL-8, growth-related oncogene (GRO)-\( \alpha \) and epithelial neutrophil-activating peptide (ENA)-78. (B) expression of the cytokines IL-1\( \beta \), TNF-\( \alpha \) and transforming growth factor (TGF)-\( \beta \). Caco-2 cells (4 x 10^5 cells/well) differentiated in six-well plates were untreated (control; C) or treated with 4 ml medium containing ETEC (\( 10^8 \) colony-forming units (CFU/ml); ETEC) K88. (K-Ba) or digested with proteases (S/P-Ba and S/P-LGG). Caco-2 cells differentiated as an inverted monolayer on Transwell filters (1.5 cm diameter) and then treated with 0.5 ml Hank’s balanced salt solution containing ETEC (\( 10^5 \) colony-forming units (CFU/ml); live probiotics (\( 10^5 \) CFU/ml) separately or simultaneously with ETEC, killed probiotics (equivalent to \( 5 \times 10^7 \) CFU) or spent probiotic supernatant fractions (equivalent to \( 5 \times 10^7 \) CFU) simultaneously with ETEC. The number of transmigrated neutrophils was quantified by myeloperoxidase assay. N-formyl-methionyl-leucyl-phenylalanine (fMLP; 1 \( \mu \)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 (\( P<0.001 \)).
Table 1. Linear regression analysis of association of neutrophil migration with chemokines and cytokines

<table>
<thead>
<tr>
<th>Chemokines and neutrophils*</th>
<th>R</th>
<th>P</th>
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<tbody>
<tr>
<td>IL-8</td>
<td>0.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Epithelial neutrophil-activating peptide-78</td>
<td>0.49</td>
<td>&lt;0.01</td>
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<tr>
<td>Growth-related oncogene-α</td>
<td>0.88</td>
<td>&lt;0.001</td>
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Cytokines and neutrophils*

| IL-1β                      | 0.81  | <0.001 |
| TNF-α                      | 0.80  | <0.001 |
| Transforming growth factor-β1 | -0.73 | <0.001 |

* Data were grouped by treatment.

The expression of chemokines may be regulated by pro-inflammatory cytokine TGF-β1, which 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-1β 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-1β and TNF-α, and negatively with TGF-β1 (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; Szekeanez 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-1β can up
regulate IL-8, GRO-α and ENA-78 (Schroder, 2000; Radack et al. 2003; Jarai et al. 2004). Conversely, TGF-β can prevent the production of TNF-α and down regulate IL-8 production (Luger et al. 1998). In our conditions, ETEC caused an up regulation of IL-1β and TNF-α, and a down regulation of TGF-B1. 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-1β was induced when LGG was added to uninfected cells. The increase of IL-1β is not completely surprising, since there is evidence that some strains of lactobacilli may up regulate pro-inflammatory cytokines (Haller et al. 2000; Ijion et al. 2004). The fact that LGG, but not B. animalis, stimulates IL-1β is in favour of the idea that individual strains of probiotics may have a different impact on the immune system, as previously indicated (Bnou-Zekri et al. 2003; Ijion et al. 2004). In addition, the results of the absence of IL-1β 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-B1 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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