PROBIOTICS POTENTIATE IL-6 PRODUCTION IN IL-1β-TREATED CACO-2 CELLS THROUGH A HEAT SHOCK-DEPENDENT MECHANISM

Natasha Reilly¹, Vitaliy Poylin¹, Michael Menconi¹, Andrew Onderdonk², Stig Bengmark³, and Per-Olof Hasselgren¹

Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA¹; Department of Pathology, Brigham Women’s Hospital, Harvard Medical School, Boston, MA²; Liver Institute, University College, London University, United Kingdom³

Running title: Probiotics and enterocyte IL-6 production

Supported in part by NIH grant R01 DK60546 (POH)

Address for correspondence: Per-Olof Hasselgren, M.D., Department of Surgery, Beth Israel Deaconess Medical Center, 330 Brookline Avenue ST919, Boston, MA 02215; Tel (617) 667-1810; Fax (617) 667-1819; e-mail phasselg@bidmc.harvard.edu
ABSTRACT

Interleukin-6 (IL-6) may exert anti-inflammatory and protective effects in intestinal mucosa and enterocytes. The influence of probiotics on mucosal and enterocyte IL-6 production is not known. We tested the hypothesis that the probiotic bacteria *Lactobacillus paracasei* and *Lactobacillus plantarum* regulate IL-6 production in intestinal epithelial cells. Cultured Caco-2 cells were treated with 1 ng/ml of IL-1β in the absence or presence of different concentrations of *Lactobacillus paracasei* or *Lactobacillus plantarum* followed by measurement of IL-6 production. The role of heat shock response was examined by determining the expression of hsp70 and hsp27, by down-regulating their expression with siRNA, or by treating cells with quercetin. Treatment of the Caco-2 cells with IL-1β resulted in increased IL-6 production, confirming previous reports from this laboratory. Probiotics alone did not influence IL-6 production but addition of probiotics to IL-1β-treated cells resulted in a substantial augmentation of IL-6 production. Treatment of the Caco-2 cells with live *Lactobacillus paracasei* increased cellular levels of hsp70 and hsp27 and the potentiating effect on IL-6 production was inhibited by quercetin and by hsp70 or hsp27 siRNA. Results suggest that probiotics may enhance IL-6 production in enterocytes subjected to an inflammatory stimulus and that this effect may, at least in part, be heat shock-dependent.
INTRODUCTION

The intestinal mucosa is an active participant in the inflammatory response to injury, sepsis, and endotoxemia and becomes the site of cytokine production during these conditions (10,21,35). In previous studies, we have been particularly interested in mucosal production of IL-6 and have found evidence that mucosal IL-6 levels are increased during endotoxemia and sepsis in mice (23,48). Although multiple cell types may contribute to increased IL-6 production in the mucosa during inflammation, experiments in IL-1β-treated cultured intestinal epithelial cells (22,30) suggest that the enterocyte is an important source of IL-6 during inflammation.

IL-6 is a pleiotropic cytokine that can have both pro- (28,32,46) and anti-inflammatory properties (51). Previous studies provided evidence that IL-6 may exert protective effects in various tissues during inflammation caused by injury and sepsis (2,40). In recent experiments in our laboratory, treatment of cultured enterocytes with IL-6 prevented cell death caused by hyperthermia (11), further supporting the concept that IL-6 can have beneficial and protective effects. Interestingly, in previous experiments we found that induction of the heat shock (stress) response potentiated the effect of IL-1β on IL-6 production in cultured enterocytes (29,34). In other studies we found that induction of the stress response in mice resulted in potentiated IL-6 production in intestinal mucosa during sepsis and endotoxemia (47). Because these effects of the stress response were associated with improved intestinal integrity (45), it is possible that increased mucosal IL-6 levels, at least in part, are responsible for the beneficial effects of the heat shock response during sepsis and endotoxemia.
Multiple studies, both in humans and experimental animals, suggest that so called probiotics exert protective effects in intestinal mucosa during various inflammatory conditions (3,4,8,9,13-15). In addition, recent studies suggest that probiotics may reduce the incidence of postoperative complications, in particular infectious complications, in patients undergoing major surgical procedures (36). It should be noticed that although multiple experimental studies support the concept that probiotics may be beneficial in the treatment of patients with inflammatory diseases of the gut, additional controlled studies are needed to more definitively identify the role of probiotics in the management of these patients. Probiotics are living bacteria with low or no pathogenicity that exert beneficial effects on the health of the host (13,15,38). Different mechanisms are probably involved in the beneficial effects of probiotics, including inhibited mucosal and enterocyte production of pro-inflammatory cytokines (18,19), inhibited activation of NF-κB (26,31), and stimulated production of the antimicrobial peptide human beta defensin-2 in intestinal epithelial cells (49). The influence of probiotics on enterocyte IL-6 production is not known but considering our previous observation that IL-6 exerts protective effects in the mucosa and enterocyte (11,45,47), the present experiments were performed to test the hypothesis that probiotics may stimulate IL-6 production in intestinal epithelial cells. Because, in a recent study, treatment of cultured enterocytes with probiotics resulted in heat shock response (31), an effect that may be caused by a soluble factor released from probiotics (43), we also examined the potential role of heat shock proteins for IL-6 production in cultured enterocytes treated with probiotics. Results suggest that probiotics can potentiate the effect of IL-1β on IL-6 production in cultured enterocytes and that this response, at least in part, is mediated by the heat shock response.
MATERIAL AND METHODS

Cell cultures

Caco-2 cells, a human colon adenocarcinoma cell line that displays enterocyte-like features in culture (33), were obtained from American Type Culture Collection (Rockville, MD). Cells were grown at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRC, Grand Island, NY) supplemented with 10% fetal bovine serum, nonessential amino acids, 6 mM glutamine, 10 mM HEPES, 10 µg/ml apotransferrin, 1 mM pyruvate, 24 mM NaHCO₃, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells, between passages 5 and 25, were seeded at a density of 100,000 cells/cm² onto 6-well tissue culture plates. Cells were grown for 72 h to approximately 80-90% confluence before use. Cells were treated with 1 ng/ml of human recombinant IL-1β (Biosource International, Camarillo, CA) or 1 ng/ml of human recombinant TNFα (Biosource International) for 20 h and IL-6 and IL-8 production was determined by measuring cytokine levels in the medium as described below. Untreated Caco-2 cells served as controls. In order to examine the influence of probiotics on cytokine production, live or heat-inactivated Lactobacillus paracasei subsp. paracasei F19 (L. paracasei, Belgian Coordinated Collection of Microorganisms, BCCM, deposition number LMG P-17806) or L. plantarum 2362 (BCCM deposition number LMG P-20606) (both strains obtained from Medipharm Inc., Kagerod, Sweden and Des Moines, Iowa, USA) were added to the culture medium at concentrations described below. In some experiments, quercetin (Sigma, St Louis, MO) was added to the culture medium at a concentration of 100 µM. Although most experiments were performed in 80-90% confluent Caco-2 cells, in a control experiment we used Caco-2 cells that had been
cultured for 3 weeks in transwell bicameral chambers to induce full differentiation as described in detail previously (24).

**Cell transfections**

In order to examine the role of hsp70 and hsp27, the expression of these genes was silenced by transfecting cells with appropriate siRNA’s. Caco-2 cells were transfected with pre-designed siRNA against hsp70 (sc-29352) or hsp27 (sc-29350). Other cells were transfected with non-specific (scrambled) siRNA. All siRNA duplexes and reagents were from Santa Cruz Biotechnology (Santa Cruz, CA) and were used according the manufacturer’s protocol.

**Preparation of bacteria**

The probiotic bacteria *L. paracasei* and *L. plantarum* were cultured in De Man-Rogosa-Sharpe (MRS) broth under anaerobic conditions at 37°C. In other experiments, *L. jensenii* (Gram-positive bacteria that are part of the normal vaginal flora) were cultured under identical conditions and were used instead of *L. paracasei* and *L. plantarum*. After culture, bacteria were collected by centrifugation (8,000 x g for 3 min), washed in phosphate buffered saline (PBS), pH 7.4, and resuspended in DMEM cell culture medium. Bacteria were added to the cultured Caco-2 cells at concentrations (determined as colony forming units, cfu) described in Results either alone or in combination with IL-1β. In some experiments, bacteria were heat-inactivated in a water bath at 80°C for 10 min or sonicated with four 20 sec bursts before they were added to the Caco-2 cells. The heat-inactivation was confirmed by culture to make certain the preparations were sterile.
In other experiments, the medium from cultured *L. paracasei* was passed through a 0.22-μm filter (Millipore, Bedford, MA) and aliquots of the filtered medium were added to the Caco-2 cells.

When the effects of *Eschericia coli* (*E. coli*) bacteria were tested, DH5α *E. coli* were first cultured in Trypticase soy broth at 37°C, washed, and grown again at 37°C under anaerobic conditions in MRS broth. The bacteria were then washed, resuspended in DMEM cell culture medium and added to the Caco-2 cells.

**Determination of IL-6 and IL-8**

IL-6 and IL-8 levels were determined in cell culture medium by commercially available ELISA kits (Endogen, Woburn, MA). The limit of detection as described by the manufacturer was 1 pg/ml for both assays.

**Western blotting**

Western blotting was performed to determine cellular levels of hsp70 and hsp27. After incubation, cells were lysed in 300 μl of lysis buffer (0.5 M Tris HCl, pH 7.4, 1.5 M NaCl, 10% NP-40, 10 mM EDTA, and 2.5% deoxycholic acid). Cell debris were removed by centrifugation at 3,800 x g for 7 min. The supernatant (whole cell lysate) was stored at -80°C until further analysis.

For Western blot analysis, aliquots of the cell lysates containing 50 μg of protein, as determined by using the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, IL), were boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol) for 7 min and then separated
by electrophoresis on a 10% Tris-glycine gradient gel (Novex, San Diego, CA). A protein ladder (See-Blue Standard, Novex) was included as a molecular weight marker. The proteins were transferred to nitrocellulose membranes (Xcell II Blot Module, Novex). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline, pH 7.6, containing 1% Tween-20 for 60 min followed by incubation with a 1:2000 dilution of mouse anti-human hsp70 (Santa Cruz Biotechnology) or a 1:2000 dilution of rabbit anti-human hsp27 antibody as primary antibodies. A goat anti-mouse IgG (Promega, Madison, WI) or a goat anti-rabbit IgG (Santa Cruz Biotechnology) secondary antibody was used at a dilution of 1:5000. The blots were then incubated in enhanced chemiluminescence reagent (ECL, Amersham Life Sciences, Buckingham, UK) and exposed on radiographic film (X-Omot AR, Eastman-Kodak, Rochester, NY).

**Determination of mRNA levels**

For determination of IL-6 mRNA levels, RNA was extracted and real-time PCR was performed as described in detail previously (50).

**Statistics**

Experiments were performed at least three times to ensure reproducibility of results. The results are reported as means ± SEM. Analysis of variance followed by Tukey’s test was used for statistical comparisons. p<0.05 was considered statistically significant.
RESULTS

In the present study, we examined the effects of two intestinal *Lactobacillus* strains because recent reports suggest that several species of *Lactobacillus* are probiotics that have protective immunomodulating properties (1,14,27), are able to prevent adhesion of pathogenic bacteria to the intestinal wall (44), and can preserve mucosal integrity (17). In initial experiments, we determined IL-6 production in cultured Caco-2 cells treated with IL-1β, live *L. paracasei* or live *L. plantarum*, either alone or in combinations. Treatment of the Caco-2 cells with IL-1β (1.0 ng/ml) for 20 h resulted in increased IL-6 production (Fig 1A), confirming previous reports from our laboratory (29,30,34). In separate experiments we found that cellular levels of IL-6 remained constant during incubation for 20 h in the absence or presence of IL-1β (data not shown), supporting the interpretation that the increased levels of IL-6 in the culture medium of IL-1β-treated Caco-2 cells reflected increased production of IL-6 rather than increased transport of cellular IL-6 into the medium. This was also supported by previous experiments in our laboratory in which treatment of cultured Caco-2 cells with IL-1β resulted in increased IL-6 mRNA levels in addition to increased amounts of the cytokine in the incubation medium (29,30,34). Treatment of the Caco-2 cells with *L. paracasei* (10^8 bacteria/ml) or *L. plantarum* (10^8 bacteria/ml) alone did not influence IL-6 production. In contrast, when the bacteria were added to IL-1β-treated cells, the IL-6 production was substantially increased, consistent with a synergistic (potentiating) effect of the probiotics (Fig 1A). The potentiating effect of *L. paracasei* was approximately 6-fold (compared with the IL-6 production in cells treated with IL-1β alone) and of *L. plantarum* approximately 2-fold.
Because the effect on IL-6 production was most pronounced for \textit{L. paracasei}, this \textit{Lactobacillus} strain was used in subsequent experiments.

Because previous reports suggest that some (but not all) effects of probiotics can be exerted by both live and heat-inactivated bacteria (18,49), we next compared the effects of live and heat-inactivated \textit{L. paracasei} on IL-6 production in Caco-2 cells. As shown in Fig 1B, the potentiating effect on IL-6 production in IL-1β-treated Caco-2 cells was similar after treatment with live and heat-inactivated \textit{L. paracasei}.

In order to test whether the potentiating effect of \textit{L. paracasei} on IL-6 production was caused by a product secreted from the bacteria, we next treated Caco-2 cells with medium from cultured \textit{L. paracasei}. Result from that experiment showed that medium from cultured \textit{L. paracasei} did not exert a potentiating effect on IL-6 production in IL-1β-treated Caco-2 cells (Fig 2A). Taken together with the results in Fig 1, these observations suggest that a factor present in live and heat-inactivated \textit{L. paracasei} potentiates IL-6 production in IL-1β-treated Caco-2 cells. In order to determine whether this factor requires intact bacterial cell walls to be effective, we next compared the effect of live intact \textit{L. paracasei} with that caused by sonicated \textit{L. paracasei}. Similar to intact \textit{L. paracasei}, sonicated \textit{L. paracasei} potentiated the effect of IL-1β and the effect of sonicated bacteria was of the same magnitude as that of intact bacteria (Fig 2B). Notably, in this experiment, IL-6 production in Caco-2 cells treated with \textit{L. paracasei} alone (third bar in Fig 2B) was higher than in the preceding experiments (compare with Fig 1A and B). This result probably reflected a day-to-day variability with regards to absolute IL-6 levels and illustrates the importance of including all study groups simultaneously when experiments are performed. Importantly, although the basal levels of IL-6 were somewhat
different, the potentiating effect of *L. paracasei* on IL-6 production was seen also in this experiment.

In order to further characterize the influence of *L. paracasei* on IL-6 production in the Caco-2 cells, we examined the effects of different concentrations of the bacteria added to the enterocytes as well as the time course for the potentiating effects. When different concentrations (10^7 – 10^9 bacteria/ml) of *L. paracasei* were added to IL-1β-treated Caco-2 cells, the maximum effect on IL-6 production was noticed for 10^8 bacteria/ml (Fig 3A). None of the concentrations tested here influenced IL-6 production when added alone to the Caco-2 cells. The potentiating effect of *L. paracasei* on IL-6 production in IL-1β-treated Caco-2 cells was noticed already after 3 h, at which time point the IL-6 production was increased approximately 2-fold, and increased progressively to be approximately 4-fold after 20 h (Fig 3B).

In order to test whether the potentiating effects of *L. paracasei* on IL-6 production in IL-1β-treated Caco-2 cells noticed here are specific for probiotics, we next treated Caco-2 cells with *L. jensenii*. These bacteria are Gram-positive (similar to *L. paracasei* and *L. plantarum*) but non-probiotic bacteria that are commonly found in the normal vaginal flora. When cultured Caco-2 cells were treated with *L. jensenii* (10^8 bacteria/ml), the IL-1β-induced IL-6 production was potentiated to a similar degree as was noticed after treatment of the Caco-2 cells with *L. paracasei* or *L. plantarum*. In this experiment, IL-6 production was 3.0 ± 0.8 pg/ml in IL-1β treated Caco-2 cells and 14.9 ± 1.2 pg/ml in Caco-2 cells treated with IL-1β + *L. jensenii*. Treatment of the Caco-2 cells with *L. jensenii* alone did not result in IL-6 production. Thus, not only probiotic Gram-positive
bacteria but other Gram-positive bacteria as well may potentiate IL-6 production in IL-1β-treated cultured enterocytes.

We next examined whether gram-negative bacteria can also potentiate the effects of IL-1β on IL-6 production in cultured Caco-2 cells. This was done by treating cultured Caco-2 cells with the *E. coli* strain DH5α. Although IL-6 production was somewhat higher in Caco-2 cells treated with both *E. coli* and IL-1β than in Caco-2 cells treated with IL-1β alone, this difference was much smaller than the approximately 6-fold potentiating effect noticed in *L. paracasei*-treated cells in the same experiment (Fig 4A).

We next determined whether *L. paracasei* can influence IL-6 production in Caco-2 cells stimulated with a cytokine other than IL-1β. Treatment of the Caco-2 cells with TNFα did not result in measurable IL-6 production and this unresponsiveness to TNFα was not influenced by *L. paracasei* (Fig 4B).

In order to test whether *L. paracasei* can potentiate the production of cytokines other than IL-6, we examined IL-8 production in cultured Caco-2 cells. Similar to previous reports (39), treatment of the Caco-2 cells with IL-1β resulted in a robust increase in IL-8 production (Fig 4C). *L. paracasei* alone or in combination with IL-1β did not influence IL-8 production in the cultured Caco-2 cells.

Most of the experiments in the present study were performed in 80-90% confluent Caco-2 cells, similar to previous reports from our laboratory in which the regulation of IL-1β-induced IL-6 production was examined (29,30). In another study from our laboratory, this model was validated by showing that IL-1β increased IL-6 production in fully differentiated Caco-2 cells grown on filters in transwell bicameral chambers (24). Here, we performed a control experiment using the same cell culture system as described
in detail previously (24) to test whether \textit{L. paracasei} can potentiate IL-1β-induced IL-6 production in fully differentiated Caco-2 cells. This also allowed us to treat the Caco-2 cells with IL-1β from the basal chamber and with \textit{L. paracasei} in the apical chamber, mimicking the situation in vivo when enterocytes are exposed to bacteria from the intestinal lumen and to circulating cytokines from the bloodstream. When this experimental approach was used, IL-6 production (determined in the basal chamber) in IL-1β-treated Caco-2 cells was potentiated by 57% by the addition of \textit{L. paracasei} (10^8 bacteria/ml) to the apical chamber (IL-6 levels being non-detectable in control cells, 11.5 pg/ml after treatment with IL-1β alone for 20 h and 18.1 pg/ml after treatment with IL-1β and \textit{L. paracasei}; means from two individual experiments). In a separate experiment, \textit{L. paracasei} alone did not influence IL-6 production in fully differentiated Caco-2 cells. Although the effect of \textit{L. paracasei} on IL-6 production in IL-1β-treated fully differentiated Caco-2 cells noticed here was less pronounced than in 80-90% confluent Caco-2 cells, the results suggest that \textit{L. paracasei} can influence the response to IL-1β with regards to IL-6 production in fully differentiated Caco-2 cells as well.

The next set of experiments was designed to elucidate the mechanism of the potentiating effect of \textit{L. paracasei} on enterocyte IL-6 production. Because Petrof et al. (31) reported recently that treatment of cultured mouse colonocytes with probiotics induced increased expression of the heat shock proteins hsp25 and hsp72 (corresponding to human hsp27 and 70, respectively) and because we found previously that induction of the stress response by hyperthermia or treatment with proteasome inhibitors potentiated the IL-6 production in IL-1β-treated Caco-2 cells (29,34), we examined the role of the heat shock response in the potentiated IL-6 production noticed here after treatment with
L. paracasei. First, we tested whether treatment of the Caco-2 cells with L. paracasei induced a heat shock response. As seen in Fig 5A and B, treatment of the Caco-2 cells with live L. paracasei resulted in increased levels of both hsp70 and hsp25 with the most pronounced effect seen for hsp70. Treatment of the cells with IL-1β did not result in changes in the heat shock protein concentrations and the combined treatment with IL-1β and L. paracasei did not influence the changes induced by L. paracasei alone.

If the heat shock response induced by L. paracasei participates in the regulation of IL-6 production in IL-1β-treated Caco-2 cells, inhibition of the heat shock response should prevent the potentiation of IL-6 production. When cells were treated with quercetin, a substance known to inhibit the induction of the heat shock response (25), the potentiating effect of L. paracasei on IL-6 production was abolished (Fig 5C). Although this result lends strong support to the notion that the effect of live L. paracasei observed in the present study is mediated by the heat shock response, one potential pitfall of the experiment using quercetin is that the drug may have non-specific effects other than inhibition of the heat shock response. In addition, it is not known from this experiment which specific heat shock protein that is involved in the effect of L. paracasei.

To address these questions, we next silenced the hsp70 and hsp27 genes by transfecting cells with siRNA’s against hsp70 and hsp25 mRNA. Treatment of Caco-2 cells with hsp70 siRNA resulted in a substantial reduction of hsp70 levels and prevented the increase in hsp70 levels induced by live L.paracasei (Fig 6A). Measurement of IL-6 production in the same cells showed that the potentiating effect of L. paracasei was substantially reduced after treatment with siRNA (from an approximately 3-fold increase in IL-6 production to an approximately 50% increase over the IL-6 production caused by
IL-1β alone) (Fig 6B). When the corresponding experiments were performed in Caco-2 cells treated with siRNA against hsp27 mRNA, the *L. paracasei*-induced increase in hsp27 levels was blocked (Fig 7A) and the potentiating effect of *L.paracasei* on IL-6 production was abolished (Fig 7B). Note that in this experiment, IL-6 production in untreated control cells was higher than in most of the other experiments in this study. Although we do not have a definitive explanation for this finding, it is consistent with a day-to-day variation of basal IL-6 production commonly seen in the current experimental model. This is why it is important to include all treatment groups and control group simultaneously in each experiment as was done in the present study. Taken together, the results in Fig 6 and 7 suggest that both hsp70 and hsp27 participate in the potentiating effect of *L.paracasei* on IL-6 production in IL-1β-treated Caco-2 cells.

In order to further assess the role of the heat shock response in the potentiating effect of *L. paracasei* on IL-6 production, two additional experiments were performed. Because we found in initial experiments, that both heat-inactivated and sonicated *L. paracasei* potentiated IL-6 production in IL-1β-treated Caco-2 cells (see Fig 1B and 2B), we tested whether heat-inactivated and sonicated *L. paracasei* induced a heat shock response (similar to live *L. paracasei*). Surprisingly, when cultured Caco-2 cells were treated for 20 h with heat-inactivated or sonicated *L. paracasei* under identical experimental conditions as used for the experiments shown in Fig 1B and 2B, hsp27 and hsp70 levels as determined by Western blotting followed by densitometry were not increased (data not shown). In the same experiment, treatment of the Caco-2 cells with live *L. paracasei* resulted in a 48% and 44% increase in hsp27 and hsp70 levels, respectively, confirming the results shown in Fig 5A and 5B. These observations suggest that additional
mechanisms (in addition to induction of the heat shock response) may be involved in the potentiating effects of \textit{L. paracasei} on IL-6 production in IL-1β-treated Caco-2 cells, at least in the potentiating effects caused by heat-inactivated and sonicated \textit{L. paracasei}.

In a second experiment designed to further assess the role of the heat shock response in the potentiating effect of \textit{L. paracasei} on IL-6 production, we compared the early time courses for \textit{L. paracasei}-induced heat shock response and \textit{L. paracasei}-induced potentiation of IL-6 expression in IL-1β-treated Caco-2 cells. In this experiment, IL-6 mRNA levels were determined by real-time PCR because we hypothesized that IL-6 mRNA levels would be influenced by IL-1β and \textit{L. paracasei} at early time points. Treatment of the Caco-2 cells with IL-1β alone resulted in an early increase in IL-6 mRNA levels noticed already after 1 h and present up to 3 h after addition of IL-1β (Fig 8). In additional experiments, IL-6 mRNA levels had returned to basal levels after 20 h (data not shown), a time point at which IL-6 protein levels were increased. These observations are in line with previous reports from our laboratory showing an early upregulation (within 1 h) of IL-6 mRNA levels in IL-1β-treated Caco-2 cells (29,30) and support the concept that IL-1β-induced IL-6 production is regulated at the transcriptional level. Importantly, in the present experiments, addition of live \textit{L. paracasei} to the Caco-2 cells resulted in a potentiation of the IL-1β-induced IL-6 mRNA levels with an almost 5-fold potentiation noticed after 1 h and an approximately 10-fold potentiation noticed after 3 h (Fig 8). Western blotting followed by densitometry showed a 23%, 51%, and 20% increase in hsp27 levels in \textit{L. paracasei}-treated Caco-2 cells at 1 h, 2 h, and 3 h, respectively (means from 2 experiments). The corresponding figures for hsp70 levels were 20%, 57%, and 33%. Thus, treatment of Caco-2 cells with live \textit{L. paracasei} resulted
in an early heat shock response that was present when an early potentiation of IL-6 mRNA expression was noticed. Although not conclusive, the results are supportive of a role of the heat shock response in the potentiating effects of live *L. paracasei* on IL-6 production in IL-1β-treated Caco-2 cells.

It should be noted that hsp27 and hsp70 were upregulated earlier (1-3 h) in the current study than in the study by Petrof et al (31) where hsp25 and hsp72 were increased 12 h after treatment of cultured young adult mouse colon (YAMC) cells with the probiotic formulation VSL#3. The reasons for the different time course of heat shock protein induction between our study and that of Petrof et al (31) are not known but may be differences in cell types being used or different probiotics used for treatment of the cells.

**DISCUSSION**

In the present study, the probiotic bacterium *L. paracasei* potentiated the effects of IL-1β on IL-6 production in cultured Caco-2 cells. In addition, treatment of the Caco-2 cells with live *L. paracasei* increased the expression of hsp70 and hsp27 and silencing of these genes with siRNA or treatment of the cells with quercetin prevented the effects of live *L. paracasei* suggesting that the potentiating effect of *L. paracasei* on IL-6 production was, at least in part, regulated by the heat shock response. Because heat-inactivated and sonicated *L. paracasei* also potentiated the IL-6 production in IL-1β-treated Caco-2 cells, without inducing a heat shock response, multiple mechanisms in addition to the heat shock response are probably involved in the potentiating effect of probiotics on enterocyte IL-6 production.
It should be noted that most experiments in the present study were performed in 80-90% confluent Caco-2 cells rather than in fully differentiated enterocytes. This experimental design was chosen to make comparisons possible with multiple previous studies in which we examined IL-6 production in approximately 80-90% confluent Caco-2 cells, including studies in which the potentiating effects of the heat shock response on IL-6 production were determined (29,30,34). Importantly, we and others found recently that although the absolute amounts of IL-6 produced from the apical and basolateral membranes in fully differentiated cultured intestinal epithelial cells may be different, treatment of the cells with IL-1β and other cytokines induced qualitatively similar responses at the apical and basolateral membranes (24,41). In a control experiment performed in the current study, *L. paracasei* potentiated the IL-1β-induced IL-6 production in fully differentiated Caco-2 cells cultured in a transwell bicameral system (although to a lesser degree than noticed in 80-90% Caco-2 cells), further validating the use of 80-90% confluent Caco-2 cells for the study of mechanisms involved in the regulation of IL-6 production by probiotics.

Although beneficial effects of probiotics have been reported in several recent clinical studies in the treatment of various inflammatory bowel diseases (3,8,9,13-15) and as preoperative treatment of patients undergoing major surgical procedures (4,36), additional well-controlled clinical studies are needed to further define the role of probiotics in the management of inflammatory bowel disease. Probiotics probably exert beneficial effects through multiple mechanisms. In general, probiotics may provide benefits by bacterial interference with intestinal pathogens (5,37) and by direct interaction with cells in the intestinal mucosa (18,19,26,31,49). In previous studies,
treatment of cultured enterocytes with probiotics resulted in inhibited activity of the transcription factor NF-kB (26,31), upregulated expression of the antimicrobial peptide human beta defensin-2 (49), improved epithelial barrier function (19), prevention of cytokine-induced apoptosis (52) and reduced production of the pro-inflammatory cytokine IL-8 (39). The apparently contradictory results in the study by Ma et al (18), i.e., reduced IL-8 production in stimulated enterocytes treated with probiotics, and the results in the present report of unchanged IL-8 production in *L. paracasei*-treated Caco-2 cells, may reflect different stimuli used to induce IL-8 production [TNFα in the study by Ma et al (18) and IL-1β in the present study], different types of probiotics [*L. reuteri* in the study by Ma et al (18) versus *L. paracasei* in the present study] and different enterocyte cell lines [T84 and HT-29 cells in the study by Ma et al (18) and Caco-2 cells in the present report].

An additional potential mechanism by which probiotics may provide mucosal protection, perhaps secondary to induction of the heat shock response, is increased production of the anti-inflammatory cytokine IL-10 (45). Interestingly, recent studies suggest that local delivery in the gut of IL-10 by genetically manipulated *Lactococcus lactis* may be beneficial in the treatment of patients with Crohn’s disease (6,42).

In the present study, we found a novel mechanism by which probiotics may exert beneficial effects, i.e., potentiated IL-6 production by enterocytes subjected to an inflammatory stimulus. This observation is important because there are multiple lines of evidence suggesting that IL-6 has mainly anti-inflammatory and protective effects in the intestinal mucosa. For example, in previous studies we found that heat shock-induced potentiation of mucosal IL-6 production in endotoxemic mice prevented mucosal injury
In other studies, IL-6 exerted cell protective effects in cultured enterocytes by inducing thermotolerance (11). Studies from other laboratories as well support the concept that IL-6 may act as an anti-inflammatory cytokine controlling both local and systemic inflammatory responses (2,40,51).

Interestingly, in the present study, L. paracasei and L. plantarum did not influence IL-6 production in the cultured Caco-2 cells under basal conditions but only in cells that were treated with IL-1β. This observation supports clinical observations that probiotics provide beneficial effects in the setting of intestinal inflammation (3,4,8,9,13-15). A lack of effect of probiotics on basal cytokine production in enterocytes has been reported by others as well (18,19).

In the present study, both live and heat-inactivated L. paracasei enhanced IL-6 production in IL-1β-stimulated enterocytes. In contrast, treatment of the cells with culture medium from the probiotics did not influence IL-6 production. These observations suggest that the effects of L. paracasei noticed in the present study were caused by a factor present in the bacteria, possibly in the bacterial wall, rather than by a secreted product. In previous reports, the effects of probiotics in cultured intestinal epithelial cells were induced by live (18) or inactivated bacteria (49) or by secreted product(s) present in the culture medium of the probiotics (31,43). These apparently conflicting results may reflect differences in probiotic bacteria as well as different intestinal epithelial cell lines being used in previous studies. It is also possible that different effects of probiotics are caused by different mechanisms. Regardless, the observations suggest that probiotics can exert their biological effects by various cell-associated and secreted factors.
Induction of the heat shock response by probiotics, as observed in the present study, is similar to a recent report by Petrof et al (31) in which treatment of cultured mouse colonic epithelial cells with the probiotics VSL #3 resulted in increased expression of hsp72 and 25. In that study (31), evidence was found that inhibition of the chymotrypsin-like activity of the proteasome may be a mechanism by which probiotics induce the heat shock response. Although we did not examine the effect of *L. paracasei* on Caco-2 cell proteasome activity in the current report, in recent experiments we found that treatment of cultured Caco-2 cells with proteasome inhibitors resulted in a heat shock response (34) similar to the effects of *L. paracasei* noticed here. Induction of the heat shock response by proteasome inhibition was reported in a number of other cell types as well (7,16,20,53).

Our results from experiments in which treatment of the Caco-2 cells with quercetin or hsp70 or hsp27 siRNAs blocked the *L. paracasei*-induced potentiation of IL-6 production strongly suggest that the heat shock response was involved in the regulation of IL-6 production. The observation that the heat shock response regulates IL-6 production in stimulated enterocytes is in line with previous reports from this laboratory in which the heat shock response was induced by other mechanisms. Thus, in previous experiments we found that when the heat shock response was induced in cultured Caco-2 cells by hyperthermia, the IL-1β-induced IL-6 production as well as the expression of IL-6 mRNA were upregulated (29). Further support for a role of the heat shock response in enterocyte IL-6 production was found in subsequent experiments in which treatment of cultured Caco-2 cells with proteasome inhibitors induced a heat shock response and potentiated IL-6 production (34). In additional experiments, induction of the heat shock
response in vivo by hyperthermia or treatment with sodium arsenite augmented mucosal IL-6 production in endotoxemic mice (47). Similar to the results in the present study, heat shock by itself did not influence enterocyte or mucosal IL-6 production in our previous studies but augmented IL-6 production that was already increased by endotoxemia in mice (47) or treatment of Caco-2 cells with IL-1β (29,34). Although the mechanisms by which the heat shock response potentiates IL-6 production in stimulated enterocytes are not fully known at present, we recently found evidence that upregulated expression and activity of the transcription factor C/EBPβ may play an important role (12).

In conclusion, the present study provides the first evidence that the probiotic bacterium *L. paracasei* may enhance IL-6 production in enterocytes subjected to an inflammatory stimulus and that the effect of live *L. paracasei* is, at least in part, heat shock-dependent. Because other studies have shown that IL-6 has anti-inflammatory and protective effects in the intestinal mucosa, the present results offer a novel mechanism by which probiotics may exert some of their beneficial effects although additional experiments will be needed to define the role of IL-6 in cell protective effects provided by probiotics.
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LEGENDS TO FIGURES

**Fig 1** The effects of IL-1β, *L. paracasei* and *L. plantarum* on IL-6 production in cultured Caco-2 cells. (A) Cultured Caco-2 cells were treated for 20 h with 1 ng/ml of IL-1β, 10^8/ml of *L. paracasei* or *L. plantarum* alone or in combinations as indicated in the figure followed by measurement of IL-6 levels in the culture medium. (B) Cultured Caco-2 cells were treated for 20 h with 1 ng/ml of IL-1β, live or heat-inactivated *L. paracasei* (10^8 bacteria/ml) alone or in combinations as indicated in the figure followed by measurement of IL-6 levels in the culture medium. Results are means ± SEM with n=3 in each group. *p<0.05 vs control (untreated cells); +p<0.05 vs cells treated with IL-1β alone by ANOVA. Identical experiments were performed three times with almost identical results.

**Fig 2** The effects of IL-1β, medium from cultured *L. paracasei*, and sonicated *L. paracasei* on IL-6 production in cultured Caco-2 cells. (A) Cultured Caco-2 cells were treated for 20 h with 1 ng/ml of IL-1β, 10^8/ml of *L. paracasei* or medium from cultured *L. paracasei* followed by measurement of IL-6 levels in the culture medium. (B) Cultured Caco-2 cells were treated for 20 h with IL-1β, intact or sonicated *L. paracasei* alone or in combinations as indicated in the figure and at concentrations identical to those used in Fig 2A, followed by measurement of IL-6 in the culture medium. Results are means ± SEM with n=3 in each group. *p<0.05 vs control; +p<0.05 vs IL-1β alone by ANOVA. Identical experiments were performed three times with almost identical results.

**Fig 3** (A) The effect of different concentrations of *L. paracasei* on IL-6 production in Caco-2 cells cultured in the absence of presence of IL-1β. Cultured Caco-2 cells were
treated for 20 h with different concentrations of *L. paracasei* alone or together with 1 ng/ml of IL-1β followed by measurement of IL-6 in the culture medium. (B) Time-course for the potentiating effect of *L. paracasei* on IL-6 production in IL-1β-treated Caco-2 cells. Cells were treated with 1 ng/ml of IL-1β or IL-1β + 10^8/ml of *L. paracasei* for various periods of time up to 20 h followed by measurement of IL-6 levels in the culture medium. Results are means ± SEM with n=3 in each group. Identical experiments were performed three times with almost identical results.

**Fig 4** (A) The effects of *L. paracasei* and *E. coli* on IL-6 production in IL-1β-treated Caco-2 cells. Cells were treated for 20 h with 1 ng/ml of IL-1β, 10^8/ml of *L. paracasei*, or 10^8/ml of DH5α *E. coli*, either alone or in combinations as indicated in the figure, followed by measurement of IL-6 in the culture medium. (B) The effects of *L. paracasei* on IL-6 production in IL-1β- and TNFα-treated Caco-2 cells. Cultured Caco-2 cells were treated for 20 h with 1 ng/ml of IL-1β or 1 ng/ml of TNFα alone or in combination with 10^8/ml of *L. paracasei*. (C) The effect of IL-1β and *L. paracasei* on IL-8 production in cultured Caco-2 cells. Cells were treated for 20 h with 1 ng/ml of IL-1β or 10^8/ml of *L. paracasei* alone or in combination as indicated in the figure followed by measurement of IL-8 levels in the culture medium. Results are means ± SEM with n=3 in each group. *p<0.05 vs control; +p<0.05 vs IL-1β alone by ANOVA. Identical experiments were performed three times with almost identical results.

**Fig 5** The influence of IL-1β and *L. paracasei* on cellular levels of (A) hsp70 and (B) hsp27. Cultured Caco-2 cells were treated for 20 h with 1 ng/ml of IL-1β or 10^8/ml of *L. paracasei* alone or together with 1 ng/ml of IL-1β followed by measurement of IL-8 in the culture medium.
*paracasei* alone or in combination as indicated in the figure. Hsp70 and hsp27 levels were determined by Western blotting. β-Actin levels were determined as loading controls. The experiment was repeated three times with almost identical results. (B) The effect of quercetin on *L. paracasei*-induced potentiation of IL-6 production in IL-1β-treated Caco-2 cells. Cells were treated for 20 h with 1 ng/ml of IL-1β or 10^8/ml *L. paracasei* in the absence or presence of 100 µM quercetin as indicated in the figure. Results are means ± SEM with n=3 in each group. *p<0.05 vs corresponding control group; +p<0.05 vs corresponding IL-1β group by ANOVA. Identical experiments were performed three times with almost identical results.

**Fig 6** The influence of reduced hsp70 levels on the *L. paracasei*-induced potentiation of IL-6 production in IL-1β-treated Caco-2 cells. (A) Hsp70 levels in cultured Caco-2 cells transfected with hsp70 siRNA or control (non-specific) RNA as described in Materials and Methods and subsequently treated with IL-1β or *L. paracasei* alone or in combination as indicated in the figure. (B) The effects of IL-1β and *L. paracasei* on IL-6 production in Caco-2 cells transfected with hsp70 siRNA or non-specific RNA. Results are means ± SEM with n=3 in each group. *p<0.05 vs control; +p<0.05 vs corresponding IL-1β group by ANOVA. Identical experiments were performed 3 times with almost identical results.

**Fig 7** The influence of reduced hsp27 levels on the *L. paracasei*-induced potentiation of IL-6 production in IL-1β-treated Caco-2 cells. (A) Hsp27 levels in cultured Caco-2 cells transfected with hsp27 siRNA or control (scrambled) RNA as described in Materials and
Methods and subsequently treated with IL-1β or *L. paracasei* alone or in combination as indicated in the figure. (B) The effects of IL-1β and *L. paracasei* on IL-6 production in Caco-2 cells transfected with hsp27 siRNA or control RNA. Results are means ± SEM with n=3 in each group. *p<0.05 vs control; +p<0.05 vs corresponding IL-1β group by ANOVA. Identical experiments were performed three times with almost identical results.

**Fig 8** The effects of IL-1β and live *L. paracasei* on IL-6 mRNA levels in cultured Caco-2 Cells. Cells were treated for 1, 2, or 3 h with IL-1β, *L. paracasei* either alone or in combination as indicated in the figure. Untreated cells served as control. IL-6 mRNA levels were determined by real-time PCR. Results are means ± SEM with n=6 in each group. *p<0.05 vs control at the corresponding time point; +p<0.05 vs IL-1β alone at the corresponding time point by ANOVA.