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Interactions of Lactic Acid Bacteria with Human Intestinal Epithelial Cells: Effects on Cytokine Production

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ABSTRACT

As a participant in the mucosal immune response, the intestinal epithelial cell must respond to a variety of stimuli, including lactic acid bacteria (LAB) consumed in the diet. The objective of this study was to compare the abilities of several strains of LAB to modulate cytokine secretion by human intestinal epithelial cell (IEC) line HT-29. Certain strains of *Lactobacillus rhamnosus, Lactobacillus delbrueckii,* and *Lactobacillus acidophilus* suppressed the production of the chemokine RANTES by stimulated HT-29 IEC, although the magnitude of this suppression varied depending on the nature of the bacterial growth medium. Similarly, specific strains showed growth condition–dependent suppression of HT-29 interleukin-8 (IL-8) production. Strain-dependent effects were also seen for the suppression of tumor necrosis factor α (TNF-α) and transforming growth factor β (TGF-β) production. The binding of several of these bacterial strains to the HT-29 cell line was also examined. Different strains were found to have differing abilities to interact with IEC, with *L. rhamnosus* R0011 being the strain that generally had the most extensive effects on HT-29 cytokine production and also bound to HT-29 IEC most effectively. Modulation of IEC cytokine production has the potential to profoundly affect the mucosal microenvironment, influencing the immune response to pathogens and other ingested antigens.

Intestinal epithelial cells (IEC) play a unique role as the initial point of contact with many pathogens and other immunological challenges. In addition to their basic barrier function, they are able to interact with cells in the gut-associated lymphoid tissue, in effect "sensing" the presence of stimuli and communicating instructions that can influence the resulting immune response *(20, 39).* Interaction with pathogens stimulates the IEC production of several mediators, including proinflammatory cytokines, inducible nitric oxide synthase, and prostaglandin (reviewed in Philpott et al. *(39)*). The ability of IEC to interact with nonpathogenic bacteria, such as the lactic acid bacteria (LAB) commonly present as part of the intestinal microflora or consumed as dietary probiotic agents, however, is less well understood.

Probiotic bacterial agents have long been defined as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" *(9).* However, recent research has suggested that probiotic bacteria have a wider range of effects, including immunomodulation, independent of their effects on gastrointestinal colonization. They may induce a heightened immune response by directly activating cells such as macrophages, B cells, and natural killer cells *(7, 13, 21, 33, 38).* The ability of lactobacilli to induce cytokine production by macrophages is now well documented *(18, 28, 29),* and lactobacilli have been shown to stimulate macrophages through the activation of NF-kB and STAT transcription factors *(32).* In light of these observations, it has been suggested that the definition of probiotic agents be elaborated to "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host" *(41),* a definition that acknowledges the actions of nonviable bacteria.

In the human gastrointestinal tract, only 30 to 40 of the approximately 400 different bacterial species present constitute 99% of the population, with *Lactobacillus* and *Bifidobacterium* spp. making up a considerable proportion of these species and with individuals having their own unique gastrointestinal microbial profiles *(34, 43).* However, under circumstances associated with stress or antibiotic treatment, the microbial balance of the gastrointestinal tract is altered, predisposing the host to various illnesses *(25, 34).* The ingestion of probiotic agents can help a host regain or maintain this balance and has potential for the prevention or treatment of ulcerative and antibiotic-associated colitis, Crohn's disease, and diarrheagenic illnesses. Beneficial effects of the ingestion of probiotic agents include the reestablishment of the normal flora and the competitive exclusion of pathogens, the production of antimicrobial substances such as bacteriocins, and the stimulation of mucin production by IECs *(27, 34).* The modulation of IEC cytokine production would also have the potential to alter the response of a host to infection.

To determine the effects of probiotic LAB on IEC cytokine production, we examined the ability of heat-killed

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strains of *Lactobacillus* spp. and *Bifidobacterium longum* to modulate cytokine production by the human IEC line HT-29. The effects of these bacteria on the proinflammatory cytokines interleukin-8 (IL-8), RANTES (regulated on activation, normal T cell expressed and secreted), and tumor necrosis factor α (TNF-α) and on the regulatory cytokine transforming growth factor β (TGF-β) were examined. The proinflammatory actions of IL-8 and TNF-α are well characterized, and their production in response to lipopolysacccharides can result in severe host damage *(17, 19)*. RANTES production by IEC is thought to play a role in later stages of inflammation in the gut mucosa because of the slower kinetics of RANTES expression *(44)*. The ability of these bacteria to attach to HT-29 IEC was also explored.

MATERIALS AND METHODS

Preparation of bacterial strains. Strains of *Lactobacillus acidophilus* (R0052), *Lactobacillus delbrueckii* subsp. *lactis* (R0187), *Lactobacillus rhamnosus* (R0011 and R0049), and *B. longum* (R0175) were obtained from the Institut Rosell Inc. (Montreal, Canada). Additional strains of *L. acidophilus* (ATCC 521), *L. delbrueckii* subsp. *bulgaricus* (ATCC 11977), *Lactobacillus plantarum* (ATCC 14917), and *L. rhamnosus* GG (ATCC 53103) were obtained from the American Type Culture Collection (ATCC; Manassas, Va.). Bacteria either were grown in deMan Rogosa Sharpe (MRS) broth (Oxoid, Nepean, Ontario, Canada) at the optimal temperature for each strain (37 to 40°C) or were rehydrated from lyophilized cultures grown in industrial formulations (Institut Rosell). Cells were collected in the stationary phase and washed repeatedly in phosphate-buffered saline (PBS) before they were used. Bacteria were heat killed in a water bath at 70°C for 1 h. All bacteria were added to IEC in tissue culture at a final concentration of 5×10^6 CFU/ml, as determined by observation of CFU and direct microscopic counts.

Cell culture and stimulation with LAB. The intestinal epithelial cell line HT-29 (ATCC no. HTB-38) was cultured in RPMI (Roswell Park Memorial Institute) 1640 (Invitrogen Gibco Life Technology, Toronto, Ontario, Canada) supplemented with 10% calf serum (Sigma Chemical Co., St. Louis, Mo.) and 5 mg of gentamycin sulfate (Sigma) per ml and incubated at 37°C under 5% CO_2. LAB were added at a concentration of 1×10^7 CFU/ml to HT-29 IEC (final concentration 5×10^5/ml). Initial preparations of LAB were made with either PBS or RPMI 1640, with all dilutions then being made in HT-29 IEC growth medium, and the appropriate cell culture controls, containing equivalent amounts of either PBS or RPMI 1640, were included. Cultures were then incubated at 37°C for the times indicated, and culture supernatants were collected for the measurement of secreted cytokines.

Measurement of cytokine production and proliferation. Enzyme-linked immunosorbent assays were used to determine the levels of IL-8, TGF-β, and RANTES produced by HT-29 cells with commercially available reagents used according to the protocols provided by the suppliers (Endogen, Woburn, Mass.; R&D, Minneapolis, Minn.). TNF-α was measured by a bioassay involving the WEHI 164 cell line, which is sensitive to TNF-mediated lysis *(30)*. HT-29 proliferative responses were measured by an MTT dye reduction assay *(6)*.

Attachment assay. Bacteria were washed in PBS and then biotinylated through incubation at 1×10^9 CFU/ml in PBS with *n*-hydroxysuccinimido-biotin (Sigma Aldrich, St. Louis, Mo.) at a concentration of 20 mg/ml at 37°C for 1 h. Biotin ester–labeled bacteria were frozen at −20°C in a 30% glycerol-in-PBS solution for later use. Labeled bacteria were then incubated with confluent IEC in a 96-well tissue culture plate for 2 h, and any nonadherent bacteria were washed free. Streptavidin-peroxidase was added to the plate, and after multiple washes the colorimetric substrate ABTS (Sigma Aldrich) was added and color development was measured at 405 nm. The results of several experiments were combined to produce normalized binding ability values by dividing all of the results within individual experiments by the results obtained for the strain showing the most extensive binding to HT-29 epithelial cells (*L. rhamnosus* R0011). In addition, the values were corrected for slight differences in biotin-labeling of bacteria between experiments.

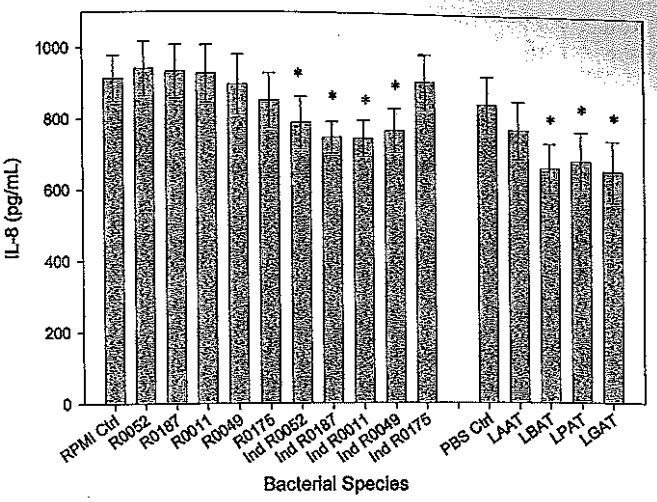

FIGURE 1. *Down-regulation of IL-8 production by HT-29 stimulated with LAB. HT-29 cells (at a concentration of 5×10^5 cells per ml) were stimulated with heat-killed LAB (at a concentration of 5×10^6 cells per ml) grown in either MRS broth (RO-#) or industrial medium (Ind RO-#), and supernatants were collected at 15 h for the measurement of IL-8 levels. Strains obtained from the ATCC (LAAT, LBAT, LPAT, and LGAT) were grown in MRS broth only. Asterisks indicate values that are significantly different from those for controls cultured without bacteria (Con-RPMI or Con-PBS) (ANOVA $P < 0.05$; $n = 10$).*

Statistical analysis. Data are expressed as means ± standard errors. All data were analyzed by analysis of variance (ANOVA), and Bonferroni's post hoc test was performed when a significant ANOVA value was obtained. A P value of ≤0.05 was considered statistically significant.

RESULTS

LAB effects on chemokine production by IEC are dependent on strain and growth medium. The effects of stimulation with different strains of LAB on IEC production of two chemokines, IL-8 and RANTES, were examined. Constitutive levels of IL-8 produced by the HT-29 cell line were between 800 and 1,100 pg/ml. Several strains were able to down-regulate the production of IL-8 (Fig. 1), including three strains of *L. rhamnosus* (R0011, R0049 [LGAT], and ATCC 53103 [GG]), as well as *L. delbrueckii* subsp. *lactis* R0187 and *L. delbrueckii* subsp. *bulgaricus* ATCC 11977 (LBAT). The effect of bacterial growth medium was examined by comparing the effects of five strains

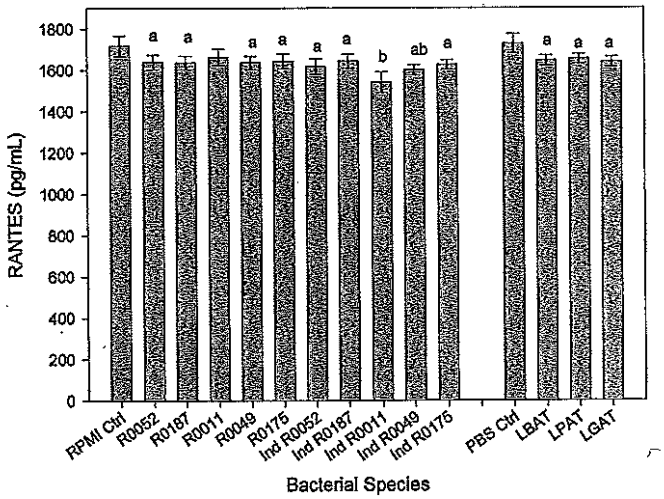

FIGURE 2. *Effects of LAB on RANTES production by HT-29 IEC costimulated with TNF-α and IFN-γ. HT-29 cells (at a concentration of 5 × 10⁵ cells per ml) were stimulated with heat-killed LAB (at a concentration of 5 × 10⁶ CFU/ml) grown in either MRS (R-#) or industrial medium (R-#) concurrently with TNF-α (50 ng/ml) and IFN-γ (100 U/ml). Strains obtained from the ATCC (LAAT, LBAT, LPAT, and LGAT) were grown in MRS broth only. Supernatants were collected at 72 h for the measurement of RANTES levels. Letters indicate values significantly different from those of controls cultured without bacteria (Con-RPMI or Con-PBS) (n = 6). Values with different letters are significantly different.*

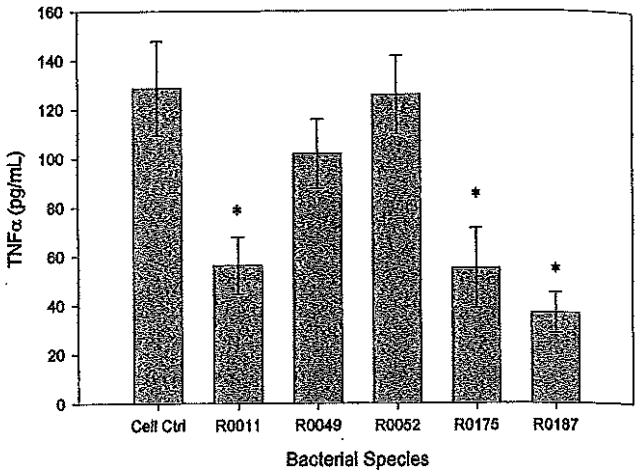

FIGURE 3. *TNF-α production by HT-29 IEC following coincubation with five strains of LAB. HT-29 cells (at a concentration of 5 × 10⁵ cells per ml) and bacteria (at a concentration of 5 × 10⁶ CFU/ml) were coincubated at 37°C for 24 h. TNF-α production was quantified by a WEHI bioassay. TNF-α production values are means ± standard errors. Asterisks indicate values that are significantly different from those for the controls* (n = 6).

(*L. acidophilus* R0052, *L. delbrueckii* subsp. *lactis* R0187, *L. rhamnosus* R0011, *L. rhamnosus* R0049, and *B. longum* R0175) grown in MRS broth with those of the same strains grown in industrial medium. Interestingly, strains R0011, R0049, and R0187 effectively decreased IL-8 production when grown in the industrial medium but not when grown in MRS broth (Fig. 1). ATCC strains were tested in MRS broth only. The concentration of LAB used (5×10^6 CFU/ml) was based on an earlier study's finding that this was an effective concentration for interactions with splenocytes *(7)*. Additional testing with *L. rhamnosus* R0011 on HT-29 IEC revealed that effective down-regulation of IL-8 production was seen at concentrations of 5×10^4 to 5×10^8 CFU/ml. The midrange concentration of 5×10^6 CFU/ml was subsequently used throughout this study.

Constitutive RANTES production by the HT-29 cell line was low (60 to 70 pg/ml). Overall, LAB alone had a minimal effect on RANTES production by HT-29 IEC, although *L. acidophilus* R0052 did slightly but significantly increase levels of RANTES production (to 85 pg/ml), an effect that was seen only after growth in industrial medium, and not after growth in MRS broth.

The optimal induction of RANTES production by epithelial cells typically requires the presence of TNF-α and gamma interferon (IFN-γ) *(23)*. When the effects of LAB were tested on HT-29 cells stimulated with these cytokines, basal levels of RANTES production were greatly increased (ca. 20-fold over baseline levels), and an effect similar to that seen for the chemokine IL-8 was revealed, with LAB moderately but consistently reducing RANTES production (Fig. 2). *L. rhamnosus* R0011 had the strongest effect, and this effect was most pronounced for preparations of R0011 grown in industrial medium rather than MRS.

Effects of LAB on TNF-α and TGF-β production by intestinal epithelial cells. Further testing of the effects of LAB on cytokine production by IEC was limited to five strains grown in industrial medium only. The level of constitutive TGF-β production by HT-29 IEC was approximately 225 pg/ml, and the level of constitutive TNF-α production was approximately 130 pg/ml. Again, certain strains had down-regulatory effects on cytokine production. TNF-α production was down-regulated by *L. rhamnosus* R0011 and also by *B. longum* R0175 and *L. delbrueckii* R0187 (Fig. 3). Of the five strains tested, *L. rhamnosus* R0011 exhibited the most potent effects on TGF-β production, causing significant down-regulation (Fig. 4). Similar results were obtained with this strain when TGF-β mRNA expression was examined by reverse transcription–polymerase chain reaction (data not shown).

To ensure that the down-regulatory effects exerted by *L. rhamnosus* R0011 on cytokine production were not due to a toxic or growth-inhibitory effect on the HT-29 cell line, the proliferative response of these IEC was also measured. Proliferation of HT-29 cells was in fact stimulated by *L. rhamnosus* R0011, confirming that reduced IL-8 production was not due to epithelial cell death (optical density [OD] values for control HT-29 cultures were 0.179 ± 0.005; OD values for HT-29 cultures incubated with *L. rhamnosus* R0011 were 0.210 ± 0.009).

Strain variation with regard to attachment to IEC. The abilities of the five strains whose effects on TNF-α and TGF-β production were tested to attach to HT-29 IEC were examined with biotinylated bacteria (Fig. 5). The different strains all bound to the IEC cell line to some extent, with *L. rhamnosus* R0011 showing the highest level of binding. It is interesting that *L. rhamnosus* R0011 was also the most

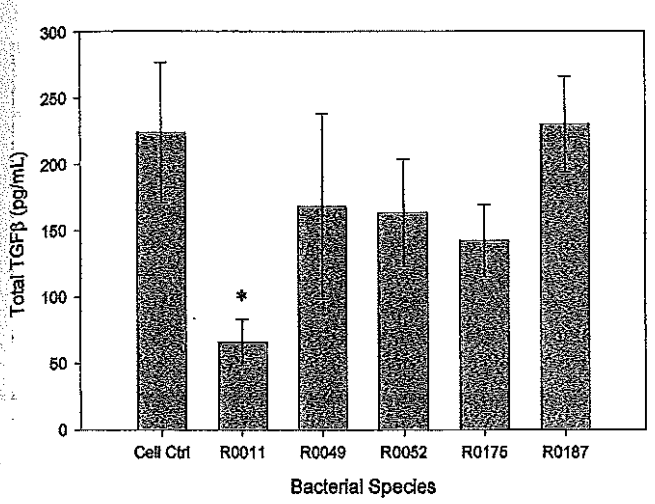

FIGURE 4. *Total TGF-β production by HT-29 IEC following coincubation with five strains of LAB. HT-29 cells (at a concentration of 5 × 10^5 cells per ml) were stimulated with heat-killed LAB (at a concentration of 5 × 10^6 CFU/ml), and total TGF-β production was measured at 18 h. TGF-β production values are means ± standard errors. Asterisks indicate values that are significantly different from those for controls cultured without bacteria (Cell ctrl;* n = *8).*

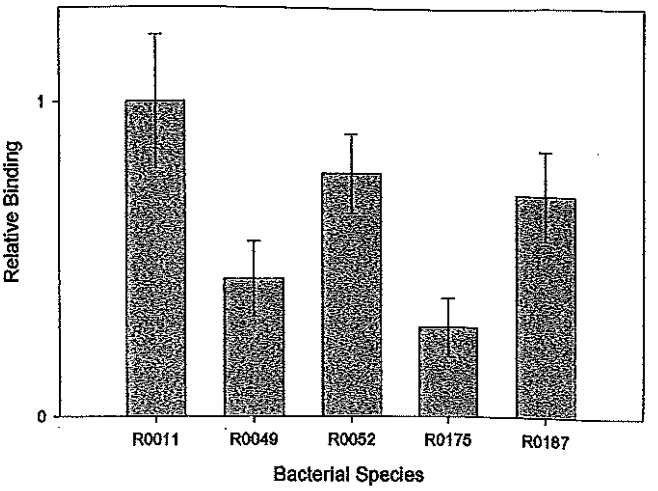

FIGURE 5. *Variation in the abilities of strains to bind to HT-29 IEC. Binding of biotinylated LAB to formalin-fixed confluent HT-29 cells was measured after 2 h of coincubation. Increased OD values reflect increased levels of bacterial binding to the epithelial cell line* (n = *6).*

potent modulator of cytokine production throughout this study. *L. acidophilus* R0052 and *L. delbrueckii* subsp. *lactis* R0187 showed intermediate binding levels, and *L. rhamnosus* R0049 showed a lower level. *B. longum* R0175 showed the lowest level of binding, and this result is consistent with its lower overall efficacy in modulating cytokine production.

DISCUSSION

Cytokine production by IEC in response to challenge with pathogenic bacteria has been the focus of much research *(4, 16, 42).* However, relatively little is known about the interactions between nonpathogenic bacteria and IEC in this context. As major constituents of the normal flora as well as through their use as probiotic agents, LAB have the potential to modulate cytokine production in the gastrointestinal mucosa. Recent findings based on a model with the human IEC line CaCo-2 cocultured with leukocytes suggest that the cytokine profile elicited from IEC by pathogens differs from that elicited by nonpathogenic bacteria, including *Lactobacillus sakei* and *Lactobacillus johnsonii (15).* These findings lend support to the idea that IEC can distinguish between pathogens and nonpathogens, possessing mechanisms for discerning bacteria that are poorly defined thus far *(39).*

We observed strain-specific differences in the ability of LAB to modulate the production of four cytokines by the HT-29 IEC line. Several strains, including *L. rhamnosus* R0011, *L. rhamnosus* R0049, *L. delbrueckii* subsp. *lactis* R0187, *L. acidophilus* ATCC 521, *L. bulgaricus* ATCC 11977, and *L. rhamnosus* GG (ATCC 53103), down-regulated IL-8 production by HT-29 IEC. Down-regulation of RANTES production was also observed, with *L. rhamnosus* R0011 having the strongest effect. This effect was only observed when HT-29 IEC were concurrently stimulated with TNF-σ and IFN-γ, suggesting that LAB alone are not effective in inducing RANTES production by IEC. Both TGF-β and TNF-α production were significantly suppressed by *L. rhamnosus* R0011, while two other strains, *B. longum* R0175 and *L. delbrueckii* subsp. *lactis* R0187, down-regulated TNF-α production but not TGF-β production. Overall, the most effective strain was *L. rhamnosus* R0011, which was able to down-regulate IEC production of all four cytokines examined, albeit to different degrees.

Changes in the abilities of these strains of LAB to interact with IEC when cultured in different growth media suggest that the culture conditions for LAB can influence the expression of cellular components involved in LAB-IEC interactions. Changes in growth conditions, including medium composition, may influence the expression of bacterial cell surface components, altering their ability to attach to IEC. Certainly, growth medium composition has been shown to influence IEC interactions with pathogens via modulation of IEC mucin production profiles *(26),* which are also modified by certain probiotic agents *(27).* Other growth factors that have been shown to influence the ability of LAB to bind to IEC include bacterial growth conditions (aerobic versus anaerobic growth), culture phase, and assay pH *(3).* For example, it has been reported that the adhesion of LAB to CaCo-2 IEC is highly dependent on growth phase, with LAB harvested in the stationary and late stationary phases showing progressively far better adherence than LAB harvested in the log phase *(3).* Interestingly, the bacterial cell growth phase has been shown to influence LAB interactions with human macrophages, inducing cytokine production, with marked differences between the effects of bacteria harvested in the exponential phase and those of bacteria harvested in the stationary phase *(14).* This observation lends further support to the suggestion that the expression of cell wall structures involved in LAB attachment to IEC begins in the stationary phase *(3).* Growth phase conditions remained constant in our study; however,

the role of the growth phase in LAB-IEC interactions merits further investigation.

Heat-killed bacteria were used in the present study, in part to prevent culture overgrowth and also to maintain defined bacterial cell numbers throughout the period of contact between IEC and LAB. While there is debate regarding the efficacy of viable versus nonviable LAB, several studies support the immunomodulatory activity of heat-treated LAB *(7, 11, 12, 28, 36, 37, 40)*. A recent study has shown that after oral delivery, both viable and nonviable *L. rhamnosus* HN001 cells are able to enhance murine phagocytic cell activity, while only viable bacteria enhance the specific antibody response to cholera toxin vaccine, suggesting that nonviable bacteria are more effective modulators of the innate than of the specific immune response *(11)*. Heat-killed LAB have been shown to be more effective inducers of monocyte TNF production than live LAB when harvested in the stationary phase *(14)*, and the results of the present study are consistent with this finding. Thus, while heat treatment may potentially have an impact on the expression and conformation of cell surface components involved in LAB-IEC interactions, there is clearly evidence of immunomodulatory activity of heat-killed LAB. This is an interesting issue, especially in light of potential applications for heat-treated LAB, and further comparison of the activity of heat-treated LAB with that of viable LAB would be valuable. To date, most examination of the innate response to LAB has focused on monocyte and macrophage activity rather than on IEC. Information regarding the ability of nonviable LAB to modulate cytokine production may also be of value with regard to probiotic agents considered for use in immunosuppressed individuals, for whom the administration of live cultures may be problematic.

Through their actions as chemokines, IL-8 and RANTES play key roles in the inflammatory response. A major function of IL-8, a C-X-C chemokine, is to stimulate transendothelial migration of neutrophils to a site of infection *(17, 23)*. RANTES is a C-C chemokine that acts as a chemoattractant for monocytes and T cells and also causes the release of histamine from basophils, actions that may contribute to the role of RANTES in exacerbating such diseases as asthma, arthritis, and inflammatory bowel disease *(10)*. The expression of RANTES tends to be delayed relative to that of other cytokines, implicating IEC as players in the later stages of the mucosal inflammatory response as well *(44)*. TNF-α also has a variety of biological activities, including the stimulation of neutrophils and macrophages, which gives TNF-α a proinflammatory role *(2)*. TNF-α can increase the secretion of IL-8 by endothelial cells, further potentiating neutrophil chemoattraction and activation.

Cytokine production by IEC may be a "double-edged sword." While the production of proinflammatory cytokines has benefits in certain situations, this activity can also contribute to local tissue damage and inflammation. Enhanced expression of IL-8, RANTES, and TNF-α in the intestinal mucosa of patients with inflammatory bowel disease has been noted *(8, 22)*. TGF-β plays primarily an immunoregulatory role, having the ability to down-regulate the production of many other cytokines, to inhibit epithelial cell division, and to promote IEC differentiation *(42)*. The diverse immunoregulatory activities of TGF-β include the maintenance of oral tolerance, the control of T-cell and dendritic-cell development and actions, and the regulation of immunoglobulin production by B cells and of monocyte and macrophage activity (reviewed in Letterio and Roberts *(24)*). While many of the activities of TGF-β are essential for the maintenance and regulation of the immune system, dysregulation of TGF-β expression can also have detrimental effects, and elevated TGF-β expression has been suggested to play a role in the fibrosis and stenosis seen in Crohn's disease and ulcerative colitis *(5)*, indicating that its actions are more complex in this context. TGF-β is also involved in tissue repair, inducing the expression of collagen$_{\alpha 1}$, fibronectin, and connective tissue growth factor, which can contribute to these complications in inflammatory bowel disease *(5)*.

The induction of these cytokines by pathogenic bacteria is part of the interaction of these bacteria with their host, and unless it is controlled appropriately, it can have damaging results. For example, IL-8 induction by *Helicobacter pylori* appears to be involved in the ability of this pathogen to produce peptic ulcer disease *(17)*. Elevated levels of IL-8 play a role in bacterial attachment to epithelial cells by up-regulating adhesion proteins *(1)*. *Shigella flexnerii* induces macrophage apoptosis, resulting in proinflammatory cytokine release from the dying cells with subsequent initiation of an inflammatory response and destruction of mucosal tissue *(17)*. If LAB can be used to counter or reduce these effects, they could play a potential protective role in situations in which excessive chemokine and cytokine production is detrimental, and they may have novel therapeutic applications in this context.

Overall, strain attachment patterns did correlate with effects on cytokine production. We used biotinylated LAB for our comparison of attachment abilities, an approach that has been successfully used to measure the surface layer protein binding of *L. acidophilus* to rat colonic mucin *(31)* and that of *Escherichia coli* to both human oral mucosa and swine enterocytes *(35)*. *L. rhamnosus* R0011, the most effective modulator of cytokine production, showed the highest level of binding to HT-29 cells. Strains *L. acidophilus* R0052 and *L. lactis* R0187 were somewhat less effective and showed lower binding levels than *L. rhamnosus* R0011. While *L. acidophilus* R0052 had less of an overall effect on cytokine production, it was the only strain that was able to induce RANTES production by HT-29 cells in the absence of exogenous IFN-γ and TNF-α. *B. longum* R0175, which modulated RANTES and TNF-α production only, showed the lowest level of binding to HT-29 cells.

A variety of factors could contribute to differences in the abilities of these strains to modulate cytokine production by epithelial cells, including differences in binding affinity between the two strains, the use of different adhesins by different strains, different levels of the same adhesin for different strains, or the binding of different strains to different ligands on the HT-29 cell line, any of which could result in the differential activation of signal transduction pathways. Currently, it appears that LAB may use a variety

of attachment mechanisms, with cell surface carbohydrates and proteins being involved in their adherence to IEC (reviewed in Naidu et al. *(34)*).

The data presented here reveal a strain-specific effect of LAB on cytokine production by IEC and provide further support for the idea that growth conditions such as medium composition can affect the ability of LAB to interact with IEC. Interactions of LAB with IEC may play a key role in their immunomodulatory effects on the mucosal immune system. Their effects on cytokine production by IEC have the potential to influence the mucosal immune response, resulting in an impact on infection and inflammation. The ability to down-regulate the production of cytokines such as TNF-α and the chemokines IL-8 and RANTES may point to potential applications for these bacteria in situations in which cytokine dysregulation at the mucosal level is the cause of damage and distress.

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