Live and Ultraviolet-Inactivated Lactobacillus Rhamnosus GG Decrease Flagellin-Induced Interleukin-8 Production in Caco-2 Cells1–3

Mariela Lopez,4 Nan Li,4 Jasmeet Kataria,4 Michael Russell,5 and Josef Neu4*

4Department of Pediatrics, University of Florida, Gainesville, FL, 32610-0296 and 5Mead Johnson Nutritional, Evansville, Indiana 47721-0001

Abstract

Probiotics are live microbial agents that can be supplemented in the treatment and prevention of gastrointestinal problems. However, in some immune-compromised populations, the administration of live microorganisms may not be appropriate. A potential alternative to live microorganisms is to inactivate them as long as the beneficial function is retained. We hypothesized that UV-inactivated Lactobacillus rhamnosus GG (LGG) could downregulate interleukin-8 (IL-8) production in intestinal epithelial cells stimulated by the pathogenic ligand, flagellin, using similar mechanisms as live LGG. Caco-2 cells were pretreated with live or UV-inactivated LGG at 1011 colony-forming units/L and stimulated by flagellin at a dose of 500 μg/mL. IL-8 production was measured by ELISA, inhibitor of κB (IkB) and ubiquitinated-IkB (Ub-IkB) expression by immunoblotting and nuclear factor (NF) κB localization by immunofluorescence staining. Flagellin induced a 17-fold increase in IL-8 production compared with control (P < 0.05), whereas pretreatment with either live LGG or UV-inactivated LGG resulted in 66 and 59% decreases, respectively, compared with the flagellin group (P < 0.05). Flagellin-induced NFκB nuclear translocation was prevented by both live and UV-inactivated LGG. Flagellin decreased IkB, which was reversed by either live or UV-inactivated LGG (P < 0.05). UV-inactivated LGG decreased Ub-IkB expression (P < 0.05), although live LGG had no effect. This study supports the concept that UV-inactivated and live LGG are equally effective in decreasing IL-8 production in the intestinal epithelium. Although the mechanism involves different pathways, both alter cytoplasmic IkB, thereby inhibiting NFκB nuclear translocation.


Introduction

The epithelium of the gastrointestinal (GI)6 tract and the GI microbiota have a mutualistic relationship where the resident bacteria aid in the metabolism of nutrients, the development of the intestinal epithelium and lymphoid tissue, and resistance to pathogenic colonization (1–4). The microbiota also play an important role in the pathogenesis of inflammatory conditions such as systemic inflammatory response syndrome (5), neonatal necrotizing enterocolitis (6,7), and inflammatory bowel diseases (2,8).

Probiotics are live microbial agents that can be supplemented to putatively replenish the GI microbiota and maintain intestinal homeostasis; however, the mechanisms of probiotic action remain poorly understood. The strain Lactobacillus rhamnosus GG (LGG) has been used as a probiotic in several studies (9) showing beneficial effects on the host, including modulation of inflammation. Our previous studies demonstrate that live or heat-inactivated LGG are able to modulate tumor necrosis factor-α (TNFα)-induced interleukin-8 (IL-8) production in the intestinal epithelium (10), but further insights into mechanisms of action are still required, including analysis of whether this response can be protective against other proinflammatory agents such as flagellin. Because of the potential impact heat treatment has on cellular integrity and protein structure, additional studies are also needed to determine whether alternative methods such as UV radiation can be used to inactivate probiotics and retain beneficial functions.

The inflammatory response in the GI tract involves the inhibitor of κB (IkB)/nuclear factor (NF)κB complex. NFκB is a nuclear transcription factor that activates the synthesis of cytokines and chemokines (11). NFκB is usually bound in the cytoplasm to the inhibitory molecule IkB, but in response to certain stimuli, phosphorylation, ubiquitination, and proteolysis of IkB occur, resulting in NFκB nuclear translocation and proinflammatory mediation (11). The GI tract appears to be tolerant to certain commensal bacteria, because they inhibit the degradation of IkB, thereby preventing NFκB translocation (12).
The potential for bacterial overgrowth in the intestine, not only of endogenous microbes but also those introduced therapeutically or prophylactically, is of concern, especially in immunosuppressed individuals (13–15). This concern could be obviated with the administration of inactive bacteria, because they have lost their ability to self-replicate. Here, the objective was to determine whether inactivated LGG could modulate inflammation in intestinal epithelial cells (specifically the Caco-2 cell line) similarly to live agents. IL-8, a proinflammatory chemokine released by intestinal epithelial cells, was used as a measurement of inflammation. High levels of IL-8 reflect an inflammatory response to the harmful effects of a destructive microorganism. Flagellin, a protein component of *Pseudomonas aeruginosa*, is a powerful proinflammatory stimulant that induces intestinal injury (16,17). We hypothesized that UV-inactivated LGG administered before the inflammatory stimulus flagellin would modulate IL-8 production by inhibiting the nuclear translocation of NFkB.

**Materials and Methods**

**Reagents.** Minimum essential medium (glutamine-free), fetal bovine serum, antibiotic antymycotic solution, and trypsin were purchased from Invitrogen Life Technologies. Glutamine, EDTA, peptide aldehyde proteinase inhibitor MG-132, and all other chemical reagents were obtained from Sigma Chemical. LGG was purchased from Valio. Antibodies to IκBα, NFkB p65, and Protein A-agarose beads were obtained from Santa Cruz Biotechnology.

**Cell culture.** Caco-2 cells were obtained from American Type Culture Collection and grown in a humidified incubator at 37°C under 5% CO₂, 95% air. For each experiment, Caco-2 cells (passage 15–28) were collected by dissociation of a confluent stock culture with 0.25% trypsin and 1 mM EDTA. Full culture media consisted of glutamine-free minimum essential medium supplemented with 20% fetal bovine serum, 4 mM L-glutamine, 100 mM penicillin, 100 mg/L streptomycin, 0.25 mg/mL amphotericin B, 1 mM sodium pyruvate, and 0.15% sodium bicarbonate. Media were changed 3 times per week. The cells were grown in triplicate in each group. Although full confluency was reached after 1 wk, experiments were initiated on d 14–15 after seeding. Previous studies in our laboratory have shown this is a time at which the cells begin to express alkaline phosphatase activity and are in an early stage of differentiation, corresponding to the upper crypt-lower villus stage of differentiation. The experiments were continued for 24–72 h as the cells progressed through more mature stages of differentiation.

**Bacteria and related preparations.** Live LGG was stored at 4°C. The activity of this powder was ~3.6 × 10¹⁰ colony-forming units (cfu)/g. Live LGG was suspended in cell culture media without antibiotics. UV-inactivated LGG were prepared by exposing LGG under a 39-W germicidal UV lamp for 5 min at a distance of 10 cm and then diluted in cell culture media without antibiotics. Before adding to each well, the LGG solution was vortexed and pipetted to ensure that a uniform dose was entered in each well. Effectiveness of the UV inactivation method was confirmed as follows: 0.2 mL of samples of LGG before and after UV exposure were diluted in PBS and plated on L agar. The plates were incubated under anaerobic conditions for 72 h at 37°C. The untreated sample was at 5.4 × 10¹⁰ cells/L. The minimal level of detection is 5000 cells/L and no colonies were detected from the 0.2-mL UV-inactivated sample.

**Expression and purification of flagellin.** Flagella were purified from *P. aeruginosa* strains grown overnight in Luria-Bertani broth. Flagella were mechanically sheared from the surface of the bacteria and collected by ultracentrifugation. The purification and extraction process was performed as previously described (18).

**ELISA for IL-8.** Cells were seeded onto 12-well plates (Costar, Corning) at a density of 2–3 × 10⁴ cells/cm². Monolayers were rinsed with PBS once and then pretreated with antibiotic-free medium containing 0 (LGG 0) or 10¹² cfu/L (LGG 10¹³) of live or inactivated LGG for 24 h. Cells were pretreated with LGG to determine whether this treatment might blunt the marked increase in IL-8 production induced by flagellin. Cells were stimulated with or without flagellin (500 µg/L) in the culture media for 48 h to ensure an adequate amount of time for stimulation. This dose of flagellin was determined by preliminary studies (18). The control group received the same cell culture media without LGG and flagellin. Cell culture media were collected and frozen at −20°C for determination of the concentrations of IL-8 by ELISA (OptEIA, Pharmingen). High Binding Extra 96-well microtiter plates (IMMULON HBX, Dynex Technologies) were used to quantify cytokines as described by the manufacturer. We read plates at 450 nm using a microplate reader (PowerWaveX Bio-TEK Instruments). Protein concentrations of whole cell lysates were measured using the BioRad DC protein assay (BioRad). IL-8 levels were normalized to standard protein concentrations.

**Immunoblotting.** Cells (6–8 × 10⁴ cells/cm²) in 100-mm-diameter dishes were cultured for 14 d. Cells were stimulated with or without flagellin (500 µg/L) in the medium for 5, 10, 15, and 30 min. These times were chosen to determine maximum IκBα degradation, which occurred at 15 min. After incubation with different concentrations (LGG 0 or LGG 10¹³) of live or inactivated LGG for 24 h, cells were stimulated with flagellin (500 µg/L) for 15 min and IκBα degradation measured by immunoblotting. The control group received the same cell culture media without LGG and flagellin. After treatment, cells were washed in PBS and suspended in lysis buffer (30 mMol/L Tris-HCL, pH 7.4, 150 mMol/L NaCl, 0.5% NP-40, 50 mMol/L NaF, 1 mMol/L Na₂VO₄, dithiothreitol, and 1% complete protease inhibitor cocktail (Roche Molecular Biochemicals)]. After cells were lysed, the solution was centrifuged at 10,000 x g; 10 min at 4°C and the cleared lysates (cytoplasmic fragments) collected. Once we determined the protein concentration of cleared lysates using BioRad DC protein assay, we separated equal amounts of protein using a 12.5% SDS-PAGE. Electrophoresed proteins were transferred from the gel to a polyvinylidene difluoride membrane. After transfer, the membrane was also stained with amido black to ensure appropriate protein transfer and equal loading of proteins. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20, incubated with primary antibody (anti-IκBα, 1:1000), and then with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000). The blot was developed using enhanced chemiluminescence (ECLPlus, Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Protein bands were quantified by densitometry using Adobe Photoshop software.

**Immunoprecipitation of ubiquitinated-IκBα.** After the cells were incubated with different concentrations (LGG 0 or LGG 10¹³) of live or inactivated LGG for 24 h, they were treated with 50 μmol/L MG-132 for 1 h before stimulation with flagellin (500 μg/L) for 15 min. The control group received the same cell culture media without LGG and flagellin. After treatment, cells were washed in PBS and suspended in lysis buffer [30 μmol/L Tris, pH 7.5, 150 μmol/L NaCl, 1.0 μmol EDTA, 1.0 μmol/EGTA, 1% Triton X-100, 1.0 mL/mouse sodium vanadate, and 1% complete protease inhibitor mixture (Roche Molecular Biochemicals)]. Cells were incubated overnight at 4°C with primary antibody (anti-IκBα, 1:1000), and then with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000). The blot was developed using enhanced chemiluminescence (ECLPlus, Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Protein bands were quantified by densitometry using Adobe Photoshop software.

**NFkB immunofluorescence staining.** Cells were cultured in an 8-well slide and treated with different concentrations (LGG 0 or LGG 10¹³) of live or inactivated LGG for 24 h, and then stimulated with flagellin (500 μg/L) for 1 h. The control group received the same cell culture media without LGG and flagellin. Cells were washed with PBS and then fixed with 3% paraformaldehyde. After blocking, a primary antibody (anti-NFkB, 1:50) was added and the slide incubated for 1 h. The slide was then incubated in the dark for 1 h with an fluorescein isothiocyanate

*Lactobacillus rhamnosus* GG decreases interleukin-8 in Caco-2 cells 2265
Results

Effect of live and UV-inactivated LGG on IL-8 production.
Flagellin alone induced a 17-fold increase in IL-8 production compared with control, whereas pretreatment with either live or UV-inactivated LGG and subsequent stimulation with flagellin decreased IL-8 production compared with the flagellin group, 66% and 59%, respectively (P < 0.05). Both forms of LGG were equally effective in downregulating a flagellin-induced IL-8 inflammatory response (Fig. 1A).

Effect of LGG on flagellin-induced IκBα degradation and ubiquitination. Whereas flagellin downregulated IκBα expression compared with control, both live and UV-inactivated LGG reversed this effect (P < 0.05) (Fig. 1B). The increase in IκBα suggests that both live and UV-inactivated LGG inhibited IκB degradation or altered its synthesis to a similar degree.

Compared with control, flagellin increased ubiquitinated-IκBα (Ub-ικB) (P < 0.05). Live LGG and UV-inactivated LGG affected Ub-ικB expression differently; live LGG did not affect the flagellin-induced increase in Ub-ικBα, whereas UV-inactivated LGG decreased Ub-ικBα expression (P < 0.05) (Fig. 1C). This suggests that live and UV-inactivated LGG effects on IL-8 production and IκBα expression may be through different pathways despite the fact that they both involve IκB and NFκB.

Effect of LGG on flagellin-induced nuclear NFκB p65 expression. Both live and UV-inactivated LGG inhibited NFκB nuclear translocation, whereas flagellin induced NFκB nuclear translocation. The control and groups pretreated with live or UV-inactivated LGG and then stimulated by flagellin showed cytosolic NFκB staining, which indicates that IκBα was not degraded and remained bound to NFκB in the cytoplasm. The cells stimulated by flagellin showed nuclear NFκB staining, indicating that IκB was degraded and NFκB translocated to the nucleus to induce inflammatory cytokine synthesis (Supplemental Fig. 1).

Discussion

Our findings show that pretreatment with either live or UV-inactivated LGG (10^11 cfu/L) significantly downregulated the 17-fold increase in IL-8 inflammatory response induced by flagellin. This study is the first to our knowledge to show that UV-inactivated LGG is as effective as live LGG in downregulating an IL-8 response in Caco-2 cells. This finding suggests the potential for safe and effective alternatives to live microbes in the prevention and treatment of conditions involving intestinal inflammation. We also found that live and UV-inactivated LGG mechanisms of action that downregulate IL-8 production merge at the IκBα and NFκB steps, despite differing effects on the ubiquitination of IκB.

Despite the recent enthusiasm for probiotic usage, there are cautionary warnings about indiscriminate manipulation of the GI microbiota during the perinatal and neonatal time periods and in individuals who may be immunosuppressed (13–15). The short-term consequences consist of bacterial translocation and sepsis, whereas long-term effects are poorly studied but need to be considered, especially in neonatal populations where the intestinal microbiota are in the early phases of becoming established (13). Consequences include the possibility of overstimulation of the inflammatory response (10) with these live organisms in compromised hosts, particularly critically ill preterm neonates (19–21) and adults (22,23), and also the potential of detrimental long-term effects, such as autoimmunity, allergies, and atopy (24,25).

The rationale for using inactivated agents stems from studies showing that interactions ofmicrobe-associated molecular patterns with toll-like receptors (TLR) and other mucosal pattern...
recognition receptors likely mediate some of the beneficial responses of probiotics. Microbial components that are TLR receptor ligands and even killed probiotics have been demonstrated to be protective if given prior to administration of agents that are injurious to the intestine (10,26–28). For example, cytosine-phosphate-guanine (29) or even TLR agonists such as lipopolysaccharide and lipoteichoic acid (28) in low doses may be effective in promoting the inhibitory actions of the intestinal epithelium in the signaling cascade of the inflammatory response (30). Use of such agents may in certain circumstances be safer than use of live agents, as previously mentioned. Further work on specific mechanisms is needed to delineate what is actually being done by the inactivated agents and if this is similar to the mechanisms of the live agents.

A previous study by our group evaluated the effects of live and heat-killed LGG on TNFα-induced IL-8 production through the NFκB pathway (10). The results showed that both forms of LGG have the ability to downregulate TNFα-induced IL-8 production in Caco-2 cells. When cells were treated with live or heat-killed LGG alone, IL-8 production increased noticeably. The effects were much less evident in cells treated with heat-killed LGG, suggesting that the heat-killed form may be safer than live LGG. The study also showed the prevention of NFκB nuclear translocation when cells were pretreated with LGG. We had similar results in our current study, where both forms of LGG modulated flagellin-induced IL-8 production and when given alone, UV-inactivated LGG did not cause a significant increase in IL-8. If inactivated probiotics provide the beneficial properties of live bacteria, they may be safer alternatives, because the dose of these agents can be readily controlled and they are less likely to establish lifelong niches that may be difficult to undo.

The flagellin of P. aeruginosa was utilized, because previous studies have demonstrated that this agent serves as a strong proinflammatory stimulus, increasing IL-8 production via activation of TLR 5. This flagellin contains a hook structure and its motility allows for it to infect intestinal epithelial cells, thereby activating NFκB (16). Although numerous intracellular signaling pathways may be involved, we focused on the IκB/NFκB pathway. Previous studies where flagellin induced IL-8 production in immature (H4 cells) and mature (T84 cells) enterocytes showed that increased production of chemokines was caused by decreased levels of IκB expression and activation of NFκB nuclear translocation (12).

Our studies of mechanisms of live and UV-inactivated LGG showed an upregulation of IκB, allowing for modulation of the inflammatory response. The IκB/NFκB complex exists in the cytoplasm of the cell, which is disrupted when the cell receives extracellular signals such as the presence of a bacterial ligand. After a signaling cascade, IκB degrades and allows NFκB to translocate to the nucleus, activating the expression of genes that allow IL-8 production to occur. This study demonstrates that LGG prevents the degradation of IκB, allowing the IκB/NFκB complex to remain in the cytoplasm and blocking the transcription of genes that lead to IL-8 production. Lack of NFκB nuclear translocation in the control and groups treated with live or UV-inactivated LGG suggests that LGG maintains the inflammatory cascade in a similar condition as the control.

Because ubiquitination of IκB is needed for its degradation, the effects of LGG on flagellin-induced ubiquitination of IκB were also studied. Our studies determined that UV-inactivated LGG decreased Ub-IκB expression, although live LGG had no effect. Studies by Neish et al. (11) suggest that the GI epithelium is tolerant to certain commensal bacteria because of their ability to decrease ubiquitination of IκB. Because our study shows that live LGG does not alter the level of IκB ubiquitination that is induced by flagellin, other possibilities such as alternative signaling pathways remain to be evaluated.

In summary, our results show that live LGG and UV-inactivated LGG significantly decrease IL-8 production in Caco-2 cells. Although further investigation is needed to understand the mechanism by which this occurs, UV-inactivated LGG may be a promising and safer alternative to live therapeutic microbial agents in at risk individuals.

**Literature Cited**


**Lactobacillus rhamnosus GG decreases interleukin-8 in Caco-2 cells** 2267


