

Soluble factors from *Lactobacillus GG* activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells

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Tao, Yun, Kenneth A. Drabik, Tonya S. Waypa, Mark W. Musch, John C. Alverdy, Olaf Schneewind, Eugene B. Chang, and Elaine O. Petrof. Soluble factors from *Lactobacillus GG* activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. *Am J Physiol Cell Physiol* 290: C1018–C1030, 2006. First published November 23, 2005; doi:10.1152/ajpcell.00131.2005.—Conditioned media from the probiotic *Lactobacillus GG* (LGG-CM) induce heat shock protein (Hsp) expression in intestinal epithelial cells. LGG-CM induces both Hsp25 and Hsp72 in a time- and concentration-dependent manner. These effects are mediated by a low-molecular-weight peptide that is acid and heat stable. DNA microarray experiments demonstrate that Hsp72 is one of the most highly upregulated genes in response to LGG-CM treatment. Real-time PCR and electrophoretic mobility shift assay confirm that regulation of Hsp induction is at least in part transcriptional in nature, involving heat shock factor-1. Although Hsps are not induced for hours after exposure, transient exposure to LGG-CM is sufficient to initiate the signal for Hsp induction, suggesting that signal transduction pathways may be involved. Experiments confirm that LGG-CM modulates the activity of certain signaling pathways in intestinal epithelial cells by activating MAP kinases. Inhibitors of p38 and JNK block the expression of Hsp72 normally induced by LGG-CM. Functional studies indicate that LGG-CM treatment of gut epithelial cells protects them from oxidant stress, perhaps by preserving cytoskeletal integrity. By inducing the expression of cytoprotective Hsps in gut epithelial cells, and by activating signal transduction pathways, the peptide product(s) secreted by LGG may contribute to the beneficial clinical effects attributed to this probiotic.

oxidant protection; stress response

PROBIOTICS are living organisms, mostly found in food supplements, which provide health benefits beyond their mere nutritive value. The concept of using bacteria to treat disease and to promote health has existed for at least 100 yr, but the evidence to support this practice has been lacking. However, now that more compelling evidence in the form of randomized and placebo-controlled clinical trials is starting to accumulate, the use of probiotics has generated much interest as a possible adjunctive therapy for numerous disease conditions (38, 41). *Lactobacillus GG* (LGG) has been used successfully in the treatment of acute and rotavirus diarrhea in infants and children (14, 28) and also in the treatment of antibiotic-associated diarrhea (3, 16). Probiotics may also prove useful in the treatment and prevention of atopic disease (16). In several animal models, the use of probiotics appears to be protective against *Cryptosporidium parvum* (1), *Helicobacter pylori* (12), and *Candida* infections (50). Finally, *Lactobacillus GG* has

been shown to decrease the level of tumor burden in a murine model of colon cancer, suggesting that this probiotic strain may also possess anti-carcinogenic activity (10).

Although probiotics appear to improve the course of many illnesses, their mechanisms of action are poorly understood (43). Attempts have been made only recently to understand the mechanisms behind their actions and interactions with the host cell. Many different possible mechanisms have been proposed, including upregulation of mucus production, improvement in epithelial barrier function, increase in IgA production, and increased competition for adhesion sites on intestinal epithelia, as well as the production of organic acids, ammonia, hydrogen peroxide, and bacteriocins, which inhibit the growth of pathogenic bacteria (6, 21, 22, 27, 29). Because of the beneficial effects of LGG demonstrated in the clinical setting, the mechanisms by which the probiotic LGG exerts its cytoprotective effects on gut epithelial cells at the cellular level were investigated.

The induction of cellular heat shock protein (Hsp) expression, which occurs after thermal stress such as fever, is a well-described mechanism by which cells are able to defend themselves against further injury. This phenomenon, known as “stress tolerance” is highly conserved throughout evolution and across all species (34). Inducible Hsps confer protection to cells in the face of a variety of different types of stress, ranging from thermal and osmotic stress to oxidative and inflammatory stressors (34). Overexpression of Hsp72 in intestinal epithelial cells has been shown to increase viability and protection against oxidative injury from NH₂Cl (32, 33), a pathophysiologically relevant reactive oxygen metabolite produced in large quantities during inflammation, when hypochlorous acid released by innate and inflammatory cells reacts with ammonia (11). In intestinal epithelial cells, the inducible Hsps, Hsp72 and Hsp25, have been shown to fortify the epithelial barrier against damage from a variety of injurious insults, thus preserving tight junction and barrier function (25, 32, 33, 39, 48).

Given their protective and beneficial effects on intestinal epithelial cell function, we hypothesized that one of the mechanisms of probiotic action may involve the induction of cytoprotective Hsps. This study demonstrates that peptides synthesized by the probiotic LGG possess the ability to induce cytoprotective Hsps in murine intestinal epithelial cells in a time- and concentration-dependent manner involving transcriptional regulation by the transcription factor HSF-1. Of

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further interest, our findings indicate that the conditioned media from LGG not only provides protection against oxidant stress and upregulates epithelial cell Hsps but also modulates signal transduction pathways.

MATERIALS AND METHODS

Tissue Culture

Young adult mouse colon (YAMC) cells are a conditionally immortalized mouse colonic intestinal epithelial cell line derived from the Immortomouse (51). The YAMC cell line was a generous gift from Dr. R. Whitehead (Vanderbilt University, Nashville, TN). These cells express a transgene of a temperature-sensitive SV40 large T antigen under control of an interferon (IFN)- γ -sensitive portion of the myosin heavy chain class II promoter (51). This feature allows YAMC cells to be cultured under nonpermissive conditions at 37°C in the absence of IFN- γ and to be propagated under permissive temperatures (33°C) in the presence of IFN- γ . YAMC cells were maintained under permissive conditions (33°C) in RPMI 1640 medium with 10% (vol/vol) fetal bovine serum, 5 U/ml murine IFN- γ (GIBCO-BRL, Grand Island, NY), 50 μ g/ml streptomycin, and 50 U/ml penicillin, supplemented with 1% ITS-X Premix (Collaborative Biomedical Products, Bedford, MA).

Before each experiment, cells were plated at a density of 2×10^5 per 60-mm dish. For RNA preparation, cells were plated at a density of 7.5×10^5 cells per 100-mm dish. After 24 h of growth at 33°C, the medium was replaced with IFN-free media, and the cells were moved to 37°C (nontransformed conditions) for 24 h to allow development of the differentiated colonocyte phenotype. Cells were treated with LGG-conditioned media (1:10 dilution directly into culture media; see below) overnight, then harvested the following day. For MAP kinase (MAPK) assays, cells were treated with LGG-CM and this was removed after 15 min and replaced with fresh RPMI media. Cells were then harvested immediately after treatment with LGG-CM for Western blot analysis (MAPK phosphorylation). MAPK inhibitors were added for 2 h before the addition of LGG-CM, and the cells were then treated for 15 min with LGG-CM. Media were then replaced with fresh RPMI and YAMC cells harvested 4 h later for Western blot analysis of Hsps or immediately after LGG-CM. This time point was chosen because this is the earliest point at which Hsp induction due to LGG-CM is usually seen. In all experiments, heat shock controls were heat shocked at 42°C for 23 min and then left at 37°C for 2 h before harvest.

Bacterial Culture and Preparation of LGG-CM

The probiotic compound, *Lactobacillus GG* (ATCC strain no. 53103), was grown to a concentration of $\sim 2 \times 10^9$ CFU/ml (as determined by colony counts) in MRS broth (Difco, Detroit, MI) for 16 h or in Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA), then centrifuged (3,000 g at 4°C for 10 min). The supernatant (conditioned media) was filtered through a 0.22- μ m low-protein-binding filter (Millex; Millipore, Bedford, MA) to sterilize and remove all bacterial cells. Aliquots of LGG-conditioned media were stored in sterile microcentrifuge tubes at -80°C until further use.

For the boiling experiments, LGG-CM was prepared as described above and then boiled for 10 min in a sand bath and allowed to cool to room temperature before use.

For the protease experiments, LGG-CM was prepared as above and then, because pepsin is active at acidic pH, was treated directly with pepsin (P7012; Sigma, St. Louis, MO) at a working concentration 0.01–0.05 mg/ml for 90 min at room temperature. LGG-CM was then subjected to sizing filtration to remove the pepsin (34.7 kDa molecular mass) with the use of a 10-kDa cutoff spin column (Centricon, Millipore) before the cells were treated. For the trypsin and proteinase K treatments, these enzymes are not active at acidic pH, so it was necessary to first adjust the LGG-CM pH from 4.0 to 8.0 using

concentrated NaOH, then LGG-CM was treated with either trypsin (no. 15400-054; GIBCO) or proteinase K (no. BP1700-100; Fisher Biotech) at a final 50 μ g/ml concentration for 90 min at 37°C. As the LGG-CM regained its activity when the pH was returned to 4.0 (see RESULTS), the pH of the treated LGG-CM was then readjusted to 4.0 using concentrated HCl and then subjected to sizing separation with the use of 10-kDa spin columns, as described above, to remove the trypsin (24 kDa) or proteinase K (28.9 kDa) before the cells were treated. YAMC cells were treated with the LGG-CM filtrate at 1:10 concentration for 16 h, and cells were harvested for Western blot analysis as described below.

For the DNA experiments, DNA was isolated from LGG bacteria using a method modified from a protocol originally used to isolate DNA from *Listeria*, another gram-positive bacillus bacteria (9). Briefly, 10 ml of LGG was grown overnight in MRS using the same method as described above, then bacteria were pelleted and resuspended in 1.0 ml of lysozyme buffer (2.5 mg/ml lysozyme, 10 mM Tris, and 20% sucrose) and incubated at 37°C for 45 min. To this, 9 ml of pronase lysis buffer (500 μ g pronase, 1% SDS, 1 mM EDTA, and 10 mM Tris) were added and incubated for an additional hour at 37°C. DNazol (Invitrogen) was then added per the manufacturer's instructions, the solution was gently mixed and then centrifuged at 10,000 g at 4°C for 10 min, and the supernatant was removed to a fresh tube. To this solution, 100% ethanol was added to allow precipitation of the DNA until the solution turned cloudy (~ 5 ml); this solution was then left to incubate at room temperature for 5 min. The solution was then centrifuged to pellet the DNA per the manufacturer's instructions, washed in 70% ethanol, and resuspended in water. DNA concentration was determined using absorbance at 260 nm with the use of a spectrophotometer (MiraiBio, Alameda, CA) and the newly isolated DNA was used immediately to treat the intestinal epithelial cells at varying concentrations. Herring sperm DNA was used as a negative DNA control. Cells were harvested the next day and processed for analysis of Hsp72 induction by ELISA (see below).

Western Blot Analysis

Cells were washed twice and then scraped in ice-cold PBS composed of (in mmol/l) 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4 , and 8 Na_2HPO_4 , pH 7.4. Cell pellets were resuspended in ice-cold lysis buffer composed of 10 mM Tris, pH 7.4, 5 mM MgCl_2 , 50 U/ml DNase and RNase, plus complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Protein concentrations were determined using the bicinchoninic acid procedure (45). Samples were heated to 75°C for 5 min after the addition of 3 \times Laemmli stop buffer and then stored at -80°C until use.

Twenty micrograms of protein per lane were resolved on 12.5% SDS-PAGE. Samples were transferred in 1 \times Towbin buffer onto PVDF membranes (Perkin-Elmer, Boston, MA) as previously described (18) and analyzed immediately. Membranes were blocked in 5% wt/vol nonfat milk in Tris-buffered saline (150 mM NaCl, 5 mM KCl, and 10 mM Tris, pH 7.4) with 0.01% (vol/vol) Tween 20 (TBS-Tween) for 1 h at room temperature. Primary antibody was added to TBS-Tween and incubated overnight at 4°C with a specific polyclonal anti-Hsp25 antibody (SPA801, Stressgen, Victoria, BC, Canada), monoclonal anti-Hsp72 antibody (SPA 810, Stressgen), or with monoclonal anti-hsc73 antibody (SPA 815, Stressgen) or actin (no. AAN01; Cytoskeleton, Denver, CO); the latter two served as controls for protein loading. Blots were washed five times in TBS-Tween before incubation with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, Ft. Washington, PA). Membranes were incubated with secondary antibodies for 1 h at room temperature and washed five times in TBS-Tween, followed by a final wash in TBS (no Tween). Membranes were then developed with an enhanced chemiluminescence system ECL reagent (Supersignal; Pierce, Rockford, IL) and developed per the manufacturer's instructions.

For the MAP kinase assays, PVDF membranes were blocked in 3% wt/vol bovine serum albumin in TBS-Tween for 1 h at room temperature. Primary antibodies were added to TBS-Tween and incubated overnight at 4°C with antibodies specific for p38 MAP kinase (MAPK) (no. 9212, Cell Signaling, Beverly, MA), phospho-p38 MAPK (no. 9211S, Cell Signaling), p44/42 ERK MAPK (no. 9102, Cell Signaling), phospho-p44/42 ERK MAPK (no. 9101S), SAPK/JNK (no. 9252, Cell Signaling), and phospho-SAPK/JNK (no. 9251S, Cell Signaling). The phosphorylated form of the kinase indicates the activated form. As positive controls, 37.7 μ M anisomycin (Alexis, San Diego, CA) was used for p38 and SAPK/JNK activation, and 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) was used for ERK1/2 activation. MAPK inhibitors (Alexis Biochemicals, Carlsbad, CA) used in this study were the p38 inhibitor SB-203580 (20 μ M), the JNK inhibitor SP-600125 (20 μ M), and the ERK inhibitor PD-98059 (50 μ M).

For the Akt experiments, YAMC cells were incubated with LGG-CM, as described for the MAPK assays, except that a concentration of 22.5% LGG-CM was used. Cells were treated for 3 min, LGG-CM-containing media were removed, replaced with fresh media, and either immediately harvested ($t = 0$) or incubated for the indicated times before harvest. As a positive control, cells were treated with 100 ng/ml murine TNF- α (Peprotech, Rocky Hill, NJ), a known activator of Akt, for 15 min before harvest. Akt was inhibited by pretreatment with LY-294002 (Cell Signaling), an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), which is upstream of Akt and necessary for Akt activation, for 1 h before LGG-CM or TNF treatment. For Western blot analysis of Akt, 15 μ g of protein per lane were resolved on 10% SDS-PAGE. Samples were transferred onto PVDF membranes, which were then blocked in 5% wt/vol nonfat milk in TBS-Tween for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies specific for the activated form of Akt with anti-Phospho-Akt (Ser473) (4051S, Cell Signaling), as well as with anti-Akt (9272, Cell Signaling) or anti-Hsc70 (SPA 815, Stressgen). Washes, incubation with secondary antibody horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch), and development of the film using the ECL reagent was performed in the usual manner as described above.

Hsp72 ELISA

YAMC cells were grown and treated with LGG DNA as described above, and the cell lysates were then prepared and tested for Hsp72 with the use of an ELISA kit (R&D Systems, Minneapolis, MN) per the manufacturer's instructions, the only difference being that the individual protease inhibitors for lysis buffer were substituted with Complete protease inhibitor cocktail (Roche, Mannheim, Germany).

RNA Isolation and RT

Cells were washed twice in ice-cold HBSS and harvested as described above, then TRIzol (Invitrogen) was added per the manufacturer's instructions and chloroform (Fisher, Fair Lawn, NJ) was added for homogenization and centrifuged (14,000 g for 15 min at 4°C) to separate phases. The aqueous phase was first removed and RNA was precipitated using isopropanol, and then washed twice with 75% ethanol. The RNA pellet was dried, dissolved in RNase-free water, and then further purified using an RNeasy spin column (Qiagen, Valencia, CA) per the manufacturer's instructions. Sample integrity was analyzed on 1% agarose gels and by absorbance at 280 and 260 nm. The cDNA was synthesized using SuperScript II RT (Invitrogen). The RT reaction was performed with the use of 3 μ g of total RNA in a total volume of 20 μ l containing the following: 1 \times first-strand buffer, 250 ng of random hexonucleotide primer, 3 μ g of RNA, 500 μ M dNTP, 10 mM DTT, 40 units of RNase Out Ribonuclease inhibitor, and 200 units of SuperScript II RT. The reaction mixture was incubated at 25°C for 10 min, then at 42°C for 50 min, and RT was inactivated by being heated at 70°C for 15 min. The

cDNA was used as a template for PCR amplification. The cDNA samples were diluted to 1:5 and stored at -20°C for further study.

Real-time PCR

Primer design. The mouse Hsp25 and Hsp72 sequences (GenBank accession numbers L07577 and BC004714, respectively) were downloaded and primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). The sense and antisense primers for mouse Hsp25 are the following: 5'-CCA TGT TCG TCC TGC CTT TC-3' and 5'-GAG GGC TGC TTC TGA CCT TCT-3'; for mouse Hsp72: 5'-GGC TGA TCG GAC GGA AGT T-3' and 5'-GGA ACG GCC AGT GCT TCA T-3'; for mouse GAPDH: 5'-GGC AAA TTC AAC GGC ACA GT-3' and 5'-AGA TGG TGA TGG GCT TCC C-3'. Real-time PCR was performed in triplicate in an iCycler with iQSYBR Green PCR supermix (both from Bio-Rad, Hercules, CA). Direct detection of PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded (ds) DNA. Three microliters of diluted (1:5) cDNA were added to bring each PCR reaction to a final volume of 25 μ l containing 1 \times SYBR Green PCR supermix and primers at a final concentration of 300 nM. The following quantification cycling protocol was used: 4 min at 95°C to activate *Taq* DNA polymerase (Bio-Rad), followed by 45 cycles of denaturation at 95°C for 15 s and annealing extension at 60°C for 15 s. The threshold cycle parameter (C_t) was defined as the fractional cycle number at which the fluorescence crossed a fixed threshold above the baseline. ΔC_t value was determined by subtracting the average GAPDH C_t value from the average Hsp25 or Hsp72 C_t value. In this study, we used the $\Delta\Delta C_t$ calculation for the relative quantitation of target without running standard curves on the sample plate. This was subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_t$ was the same as the standard deviation of the ΔC_t value. The relative change in YAMC RNA (target gene) compared with the GAPDH endogenous control was determined by the formula relative change = $2^{-\Delta\Delta C_t}$, per the manufacturer's recommendations (User Bulletin no. 2, ABI PRISM 7000 Sequence Detection System; PE Applied Biosystems).

Electrophoretic Mobility Shift Assay

Cells were either treated with LGG-conditioned media (LGG-CM) for the times indicated or heat shocked as described above. Whole cell extracts were prepared in lysis buffer composed of 25% vol/vol glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 20 mM HEPES (pH 7.4), 0.5 mM PMSF, 1 mM Na₃VO₄, 1 mM β -glycerophosphate, and Complete protease inhibitor cocktail (Roche Molecular Biochemicals) by freezing once in a dry ice/alcohol bath, thawing on ice, shearing gently with a pipette tip, followed by centrifugation at 50,000 g for 5 min at 4°C. The supernatant was then removed and stored at -80°C. Labeling reactions were performed by mixing 100 ng of heat shock element (HSE) oligonucleotide [containing four tandem inverted repeats of the heat shock element (5'-nGAAn-3'): CTAGAAGCTTCTAGAAGCTTCTAG] with 20 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and 50 μ Ci of [γ -³²P]ATP (Perkin-Elmer) in 1 \times kinase reaction buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 0.1 mM EDTA, 5 mM DTT, and 0.1 mM spermidine). The kinase reaction was allowed to incubate for 60 min at 37°C, and then labeled oligonucleotide was separated from free nucleotide using G50 spin columns (Amersham Biosciences, Piscataway, NJ) by following the manufacturer's instructions. Annealing of labeled oligo and unlabeled oligo strands was performed at 95°C for 5 min, then allowed to cool slowly overnight. Ten micrograms of whole cell and nuclear extract were then mixed with [γ -³²P]ATP-labeled HSE oligonucleotide (50,000 cpm) and 0.5 μ g poly(dI-dC) (Roche, Mannheim, Germany), 10 μ g of BSA in 1 \times binding buffer (20 mM Tris, pH 7.8, 100 mM NaCl, 1 mM EDTA, and 10% vol/vol glycerol). The binding reaction was allowed to incubate for 25 min at room temperature. Samples were then analyzed on a 4%

nondenaturing polyacrylamide gel run in $0.5\times$ Tris-borate-EDTA. The gels were dried and autoradiographed to detect DNA protein complexes. For supershift experiments, YAMC cells were incubated with LGG-CM and then $1\ \mu\text{g}$ of rat monoclonal anti-HSF-1 antibody (SPA 950, Stressgen, Victoria, BC, Canada), $1\ \mu\text{g}$ of rat monoclonal anti-HSF-2 (SPA 960, Stressgen), or $1\ \mu\text{l}$ of rabbit preimmune serum was preincubated with cell extracts at 25°C for 30 min before the HSE binding reaction. After this preincubation, the binding reaction and analysis were performed as usual.

Microarray

RNA was prepared as described above and then subjected to one additional purification step using RNeasy Mini Kit (Qiagen) per the manufacturer's instructions. The integrity of the RNA sample was evaluated with the use of a Bioanalyzer (model 2100, Agilent Technologies, Palo Alto, CA) and purity/concentration was determined using a Gene Spec III spectrophotometer (MiraiBio). Only RNA with a 260 nm-to-280 nm ratio between 1.8 and 2.0 was used. A murine Affymetrix microarray chip (no. 430A), containing 19,000 murine genes was run in duplicate, and then murine Affymetrix microarray chip no. U74Av2 containing 12,000 murine genes but recognizing different probe sets was used to further confirm the results obtained with the Mouse 430A microarray chip. Data were analyzed with Genechip Operating Software (version 1.0; Affymetrix). In each case, LGG treatment was compared with mock treatment controls. Results are expressed as the relative change of treated cells compared with controls as calculated using Genespring software (version 4.2.1, Silicon Genetics, Mountain View, CA). Statistical analysis was performed with the use of D chip software (23). Differentially expressed genes were selected based on the following thresholds: relative difference >1.5 fold, absolute difference >100 signal intensity units, and statistical difference of $P < 0.05$. Data from the Affymetrix 430A microarray chips has been deposited in the GEO databank and can be accessed at <http://www.ncbi.nlm.nih.gov/geo/> series entry (GSE1940).

Chromium Release Assay for Cell Viability

YAMC cells were grown in 24-well plates and either left untreated (control), or treated with LGG-CM for 1 h and then the media were replaced and cells were left overnight at 37°C , 5% CO_2 incubator. Cells were then loaded with ^{51}Cr ($50\ \mu\text{Ci/ml}$; Sigma) for 60 min, washed, and incubated in the media with $0.6\ \text{mM}$ of the oxidant NH_2Cl to induce cell injury. After 60 min, the media were harvested and the ^{51}Cr remaining in the cells extracted with $1\ \text{N HNO}_3$ for 4 h. ^{51}Cr in the released and cellular fractions was counted by liquid scintillation spectroscopy. ^{51}Cr released was calculated as the amount released divided by the amount released plus the cellular remainder. The data were compiled and analyzed using InStat software (GraphPad, San Diego, CA), and comparisons were made using a paired Student's *t*-test.

For silencing of LGG-induced Hsps, YAMC cells were plated and allowed to grow for 24 h in complete medium. Twenty-five silencing oligonucleotides were designed for Hsp25 (corresponding to bases 1,266–1,290 and 1,503–1,527 of mouse Hsp25 GenBank accession no. L07577) or Hsp72 (bases 1,691–1,715 of human Hsp72 GenBank accession no. M11717) with the use of RNAi designer software (Invitrogen). For each well, sufficient oligonucleotide for a final concentration of $20\ \text{nM}$ (in $500\ \mu\text{l}$) was added to $100\ \mu\text{l}$ of Opti-Mem medium (Invitrogen, Grand Island, NY) and mixed with $0.4\ \mu\text{l}$ of SilentFect reagent (Bio-Rad) in $100\ \mu\text{l}$ Opti-Mem, and allowed to complex for 20 min at room temperature. Medium was removed from the cells, and the oligo/SilentFect mixture in Opti-Mem was added to the well and allowed to sit for 60 min. At this time, $300\ \mu\text{l}$ of complete medium were added and the cells were allowed to grow for either 48 h [for Hsp72 small interfering RNA (siRNA)] or were repulsed with siRNA after 24 h (for Hsp25 siRNA) and allowed to grow for an

additional 24 h. The cells were treated with LGG-CM as above 1 day before chromium loading and NH_2Cl injury as above. When inhibitors of MAPKs were used, the cells were treated with PD-98059 ($50\ \mu\text{M}$), SB-203580 ($20\ \mu\text{M}$), or SP-600125 ($20\ \mu\text{M}$) for 2 h before the addition of LGG-CM. After 1 h, LGG-CM was added, and cells were left in LGG-CM-containing media for 1 h. The cell culture media containing inhibitors and LGG-CM was then replaced with fresh media (i.e., no inhibitors or LGG-CM), and cells were returned to the incubator overnight before chromium loading and injury.

G/F Actin Assay

Confluent YAMC cell monolayers were switched to 37°C in IFN- γ -free medium and treated with LGG-CM for 1 h, after which the media were replaced or left untreated (control). Cells were left overnight and then treated with the oxidant NH_2Cl ($0.6\ \text{mM}$ for 30 min) to induce cell injury (32, 33). Cells were rinsed in PBS, harvested, centrifuged ($14,000\ \text{g}$ for 20 s at room temperature), and the pellets were resuspended in $200\ \mu\text{l}$ of 30°C lysis buffer [$1\ \text{mM}$ ATP, $50\ \text{mM}$ PIPES, pH 6.9, $50\ \text{mM}$ NaCl, $5\ \text{mM}$ MgCl_2 , $5\ \text{mM}$ EGTA, 5% (vol/vol) glycerol, 0.1% (vol/vol) Nonidet P-40, Tween 20, and Triton X-100, containing complete protease inhibitor cocktail]. Cells were homogenized by being gently pipetted up and down 10 times, incubated at 30°C for 10 min, and then subjected to centrifugation at $100,000\ \text{g}$ for 60 min (30°C). Supernatants were removed for determination of G-actin, and pellets (containing F-actin) were resuspended in $200\ \mu\text{l}$ of 4°C distilled water with $1\ \mu\text{M}$ cytochalasin D and left on ice for 60 min. (This treatment depolymerizes the F-actin fraction so that only the monomeric 45 kDa form will be observed on the Western blots.) Afterward, $20\ \mu\text{l}$ of each extraction were removed, Laemmli stop solution was added, and the samples were heated to 65°C for 10 min. Samples were resolved on 12.5% acrylamide gels by SDS-PAGE and immediately transferred to PVDF membranes (see *Western blot analysis* for further details). After transfer, immunoblot analysis of actin was performed using a polyclonal anti-actin antibody (Cytoskeleton).

RESULTS

LGG-CM Induces Hsps

Conditioned media from probiotic LGG induced Hsp25 and Hsp72 expression in cultured murine colonic YAMC cells in a time-dependent fashion, with Hsp25 expression beginning after 18–20 h (Fig. 1B) and Hsp72 being expressed earlier, first appearing at 4–6 h (Fig. 1C). During the course of this treatment, expression of the constitutively expressed heat shock cognate 73 (Hsc73) did not change, indicating that the effect of LGG-CM was specific to inducible forms of Hsp. The epithelial cells responded to the LGG-CM, with the most robust response observed with 1:10 dilution (Fig. 1A). The Hsp-inducing effects of live LGG bacteria are also shown (Fig. 1D).

Unlike the rapid response seen with thermal stress, which induces Hsp within a matter of 2 h in YAMC cells, the response to LGG-CM took considerably longer. Hence, we were interested in further investigating the mechanism behind LGG-CM-induced Hsp expression, which appeared different from thermal stress.

LGG-CM Induction of Hsps Occurs Through a Transcriptional Mechanism

With the use of real-time PCR, it was found that mRNA levels for both Hsp25 and Hsp72 increased after LGG-CM treatment, suggesting that the induction of these two Hsps by LGG-CM could be transcriptional in nature (Fig. 2). To further investigate the nature of Hsp induction by LGG-CM, electro-

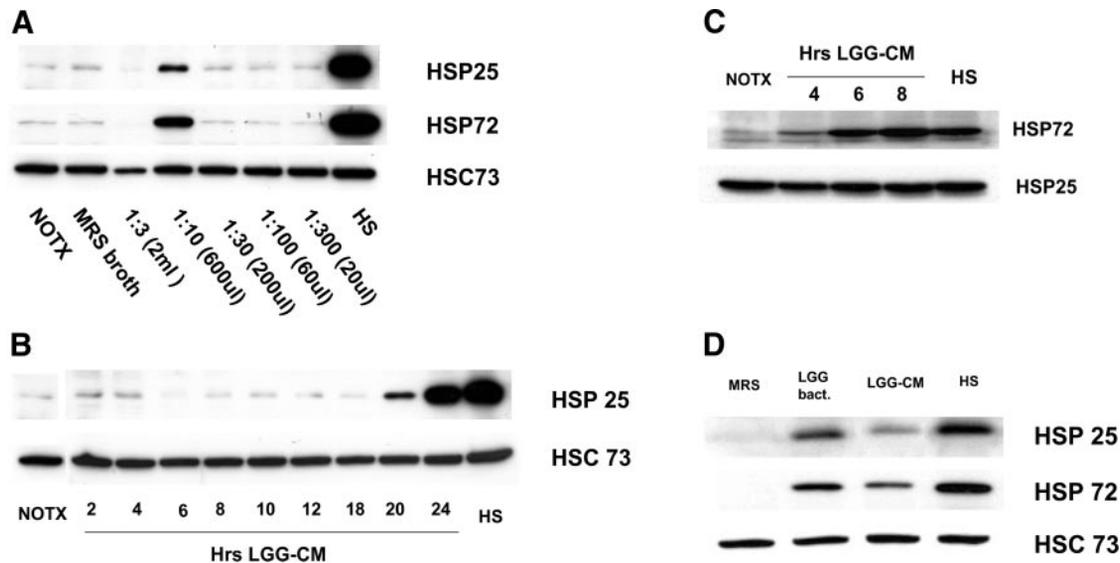


Fig. 1. *Lactobacillus GG*-conditioned media (LGG-CM) induce young adult mouse colon cells (YAMC) heat shock protein (Hsp)25 and Hsp72 in a time- and concentration-dependent fashion. YAMC cells were untreated (NoTx), treated with vehicle control (MRS) broth (1:10 dilution), or treated with varying dilutions of LGG-CM for 16 h (A) or with a 1:10 dilution for varying times (B) and (C). Hsp protein expression was determined by Western blot analysis. As a positive control, cells were heat shocked for 23 min at 42°C, followed by a 2-h recovery (HS). The effect of live bacteria (LGG bact.) is also shown (D). In this case, bacteria were left to coincubate with YAMC cells for 1 h, washed off, and cells were harvested 16 h later. Images shown are representative of 3 separate experiments.

phoretic mobility shift assays were performed (Fig. 3). LGG-CM treatment induces binding of HSF-1 as early as 5 min after exposure, decreasing by 3 h, supporting the idea that induction of Hsp expression by LGG-CM is at least partly transcriptional in nature. Binding of HSF-1 occurs within the first hour of exposure to LGG-CM, and supershift analysis demonstrates a shift of the HSF-HSE complex using anti-HSF-1 but not anti-HSF-2 antibodies, indicating that HSF-1 is the principal transcription factor involved (Fig. 3).

Hsps Are the Most Highly Upregulated Genes in Response to LGG-CM

After having established that LGG-CM treatment induces Hsps and that the mechanism behind Hsp induction by LGG-CM in epithelial cells is at least in part transcriptional in nature, the ability of LGG-CM to induce other genes and the magnitude of

the upregulation of Hsps was investigated. RNA from LGG-CM-treated and MRS (mock)-treated cells were compared using an Affymetrix gene chip cDNA microarray containing 19,000 murine genes. The most dramatically upregulated genes in response to LGG-CM treatment are the Hsp genes (Table 1). To confirm these findings, an additional gene chip containing 12,000 murine genes and using different probe sets was used and, again, Hsps were found to be the most upregulated genes in response to LGG-CM treatment (data not shown). Table 1 lists the 10 genes in intestinal epithelial cells upregulated to the greatest degree in response to LGG-CM treatment.

LGG-CM Exposure Causes Rapid Hsp Induction and Activates MAPK, Akt

Next, it was determined whether induction of Hsps could be initiated after a transient exposure to LGG-CM. In other words,

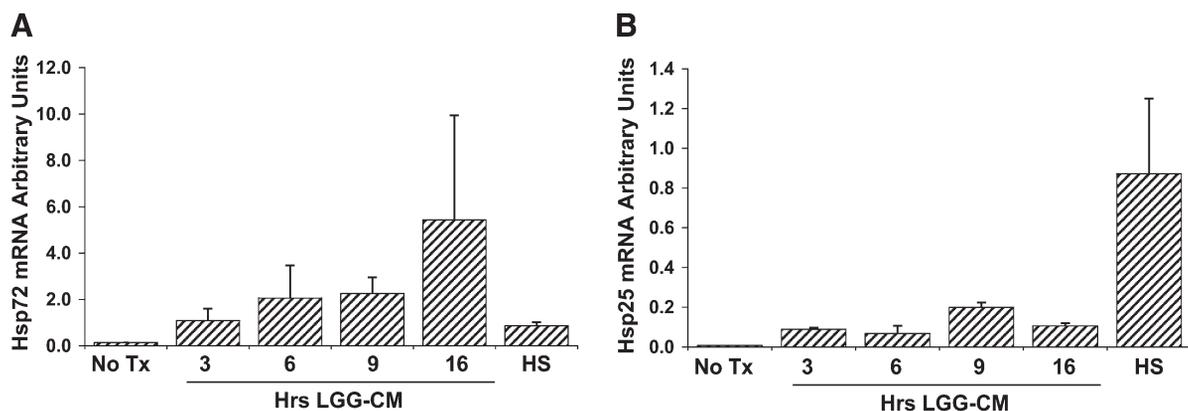


Fig. 2. LGG-CM increases Hsp25 and Hsp72 mRNA. YAMC cells were NoTx, treated with a 1:10 dilution of LGG-CM for several hours, or heat shocked (HS), as described in MATERIALS AND METHODS. RNA was harvested and analyzed for Hsp25 and Hsp72, as well as GAPDH by real-time PCR, as described in MATERIALS AND METHODS. Data presented represent means \pm SE for 3 separate experiments. Induction of Hsp mRNA was normalized to the GAPDH signal of each sample.

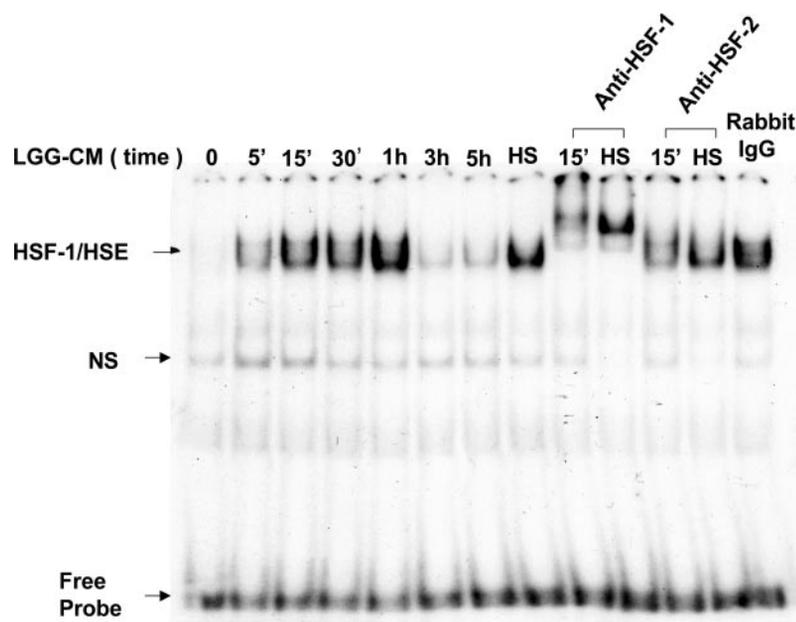


Fig. 3. LGG-CM activates heat shock transcription factor-1 (HSF-1). YAMC cells were treated with 1:10 dilution of LGG-CM for varying times (0–5 h) or heat shocked (42°C for 23 min) and immediately harvested, and whole cell extracts were prepared for analysis of the transcription factor HSF-1 activation by electrophoretic mobility shift assays (EMSA), as described in MATERIALS AND METHODS. To test for the HSF isoform activated, after 15 min of treatment with LGG-CM, the samples were also incubated with anti-HSF-1 (lane 9) or anti-HSF-2 (lane 11) antiserum as well as preimmune serum (far right lane) to detect any alteration in migration of the activated HSF-heat shock element (HSE) complex. NS, non-specific binding of the HSE, which occurs even in unstimulated cells. Image shown is representative of 3 separate experiments.

if the LGG-CM were removed and replaced by regular medium early in the course of treatment, would this transient exposure be sufficient to cause induction of Hsps, or did Hsp induction require prolonged exposure to the LGG-CM? Cells were exposed to LGG-CM for short periods of time (0–30 min), the LGG-CM-containing medium was removed, and cells were analyzed for Hsp production after 16 h (Fig. 4A). Even exposure times of a few minutes were sufficient to induce a robust response of Hsp induction, indicating that the time required to initiate the signal to the epithelial cells for the induction of Hsps is very short. Given the rapidity of the response, these data suggest that early stimulation of signal transduction pathways may be involved. To investigate this possibility, cells were treated with LGG-CM for 15 min and then analyzed for MAPK activation. Many protein kinases are known to be activated by stresses, such as LPS, TNF- α , heat, ultraviolet radiation, chemicals, and osmotic shock, and several of these kinases belong to the MAPK family (6a). Because members of the MAPK family have been shown by others, albeit in a different context, to be modulated by LGG treatment in the

same cell line used in our studies (53), the effects on this group of kinases were chosen as a readout for signal transduction activation. Even after short exposure times, differences in kinase activation between treated and untreated cells were apparent (Fig. 4B). Pretreatment of cells with LGG-CM alone activates all three MAPKs investigated. Although there is a baseline level of activated ERK1/2 in our YAMC cells, the activation of ERK1/2 by LGG-CM was almost as robust as activation by the phorbol ester PMA, whereas LGG-CM treatment resulted in a clear but less dramatic activation of p38 and JNK than was seen with anisomycin (known potent stimulator of p38 and SAP/JNK activation). Inhibitors against all three MAPKs investigated were used to determine whether activation of a MAPK pathway was required for Hsp induction by LGG-CM. Exposure of YAMC cells to inhibitors against p38 and JNK before LGG-CM treatment resulted in blockade of Hsp72 expression, thus confirming a likely role for MAPK signaling pathways in the induction of Hsps by LGG-CM in epithelial cells (Fig. 4C). Densitometry of these immunoblots indicates that PD-98059 had a more modest effect than the p38

Table 1. Heat shock proteins are among the top 10 upregulated genes in colonic epithelial cells after treatment with LGG-CM, as determined by Affymetrix chip DNA microarray analysis

GenBank Accession No.	LGG-CM vs. NoTx, -Fold Change	Gene Description	P Value
AW763765	68.59	Mouse inducible Hsp70 3	0.00002
M12573.1	36.76	Mouse inducible Hsp 70 kDa 1 (Hsp68)	0.00002
U03561.1	22.63	Mouse Hsp27 internal deletion variant b	0.00002
AF071754.1	13.93	Mouse DNA cytosine methyltransferase	0.000492
AK013777.1	13.00	Protein tyrosine phosphatase, nonreceptor type 21	0.00003
BC025911.1	12.13	Mouse sorting nexin 6	0.000241
NM_013560.1	11.31	Mouse Hsp25	0.00002
L07264.1	11.31	Mouse heparin-binding EGF-like growth factor precursor	0.00002
BF320427	11.31	RIKEN cDNA 5430423O14 gene (EST, gene function unknown)	0.001832
L25109.1	10.56	Mouse lissencephaly gene partial cds	0.000147

Young adult mouse colon (YAMC) cells were treated with conditioned media for *Lactobacillus GG* (LGG-CM) and subjected to DNA microarray analysis as described in MATERIALS AND METHODS. NoTx, no treatment; Hsp, heat shock protein. Hsp72 is synonymous with murine inducible Hsp70. The relative (-fold) increase in gene expression for the top 10 genes is listed in column 2. The rest of the data have been deposited in the GEO databank and can be accessed at <http://www.ncbi.nlm.nih.gov/geo/series> entry (GSE1940).

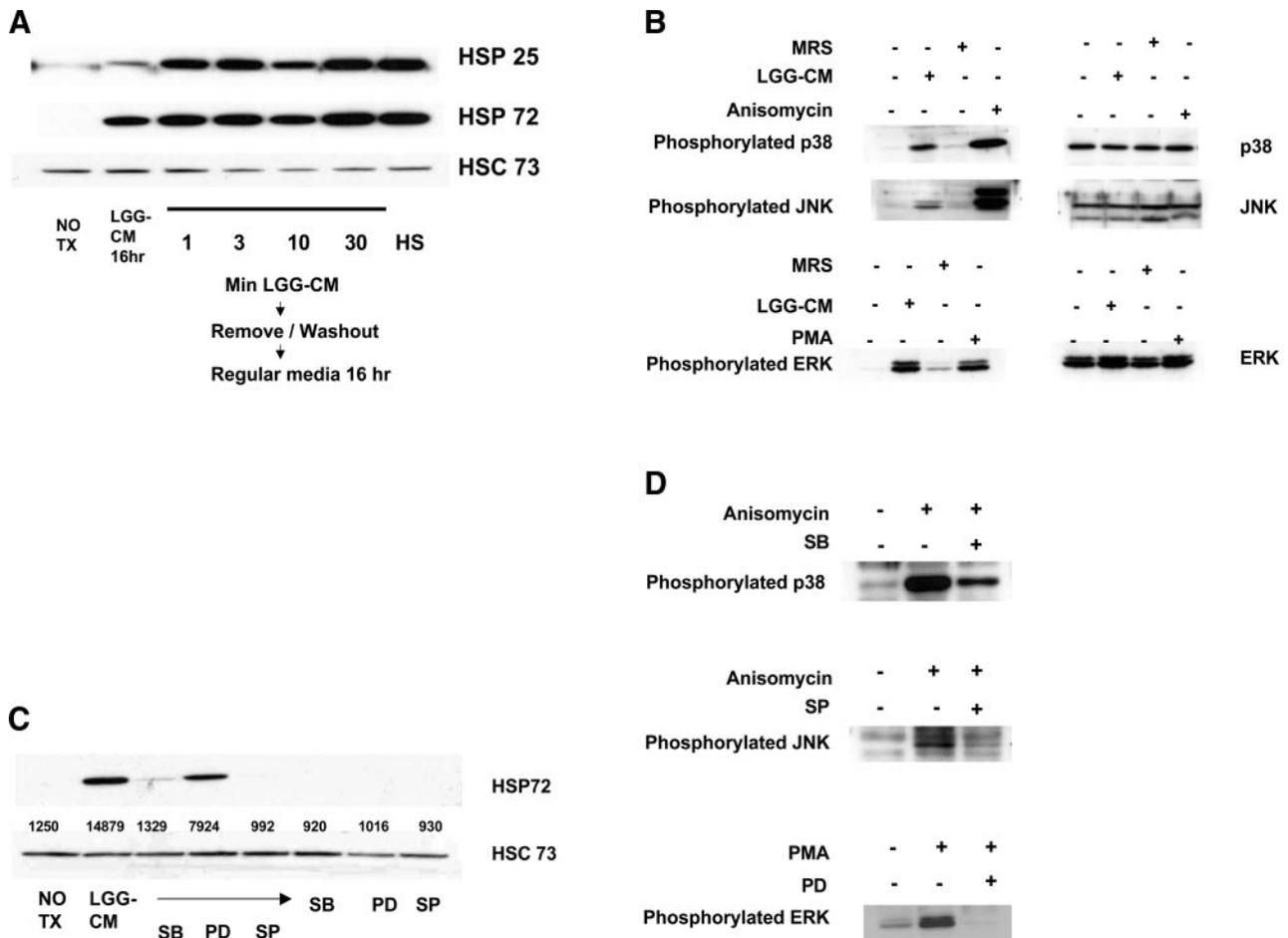


Fig. 4. LGG-CM requires only transient exposure to induce Hsps and rapidly activates MAP kinases. YAMC cells were treated with vehicle control (MRS) or with 1:10 dilution of LGG-CM for varying times, followed by removal and replacement with regular medium for 16 h overnight ("O/N"). *A*: Hsp induction was determined by Western blot analysis, as described in MATERIALS AND METHODS. Image shown is representative of 3 separate experiments. *B*: YAMC cells were treated with 1:10 dilution of LGG-CM for 15 min, and then MAP kinase activation was determined by Western blot analysis with specific antibodies to activated ERK1/2, p38, or SAPK/JNK. Samples were always analyzed for total expression of these kinases using antibodies which recognize both active and inactive forms. Phorbol 12,13-myristate acetate (PMA) was used as a positive control to activate ERK1/2, and anisomycin was used as a positive control to stimulate p38 and JNK. Image shown is representative of 3 separate experiments. *C*: YAMC cells were treated with inhibitors against MAP kinases for 2 h (20 μ M p38 inhibitor SB-203580, 20 μ M JNK inhibitor SP-600125, and 50 μ M ERK inhibitor PD-98059). Cells were then treated for 15 min with LGG-CM, followed by removal and replacement with regular medium. Cells were harvested 4 h later to assess Hsp72 induction by Western blot analysis as described in MATERIALS AND METHODS. Densitometry for the corresponding lanes was determined using NIH Image J software and is indicated by the numbers below each lane of the Hsp72 autoradiogram. The inhibitory activity of the MAP kinase inhibitors used in *C* was tested, and this is shown in *panel D*. Image shown is representative of 3 separate experiments.

and JNK inhibitors on inhibiting Hsp72 expression, suggesting that ERK plays a lesser role. The inhibitory activity of the MAPK inhibitors used for their respective kinases was verified, and this is shown in Fig. 4*D*.

Finally, cells were treated with LGG-CM and examined for activation of Akt, a gene which plays an important role in cell survival. As has been shown by others studying live bacteria (53), Akt is activated by LGG-CM and this effect is inhibited by treatment with the PI3-kinase inhibitor LY-294002 (Fig. 5).

LGG-CM Protects Epithelial Cells Against Oxidant Damage

Because LGG-CM upregulates inducible Hsps, functional assays were undertaken to determine whether this phenomenon bears physiological significance. Normally produced when hypochlorous acid released from innate immune cells reacts with ammonia, the oxidant NH_2Cl affects epithelial cells by causing cytoskeletal disruption, impaired membrane transport,

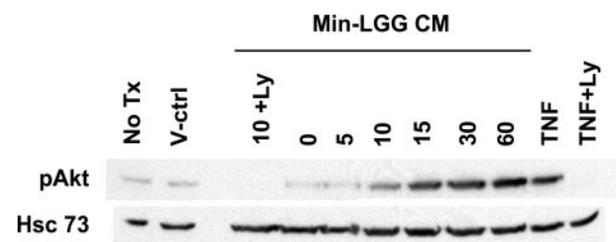


Fig. 5. Transient exposure to LGG-CM activates Akt kinase. YAMC cells were treated with LGG-CM at a concentration of 22.5% for 15 min, then media were removed and cells were harvested for varying times as indicated. V-ctrl, cells treated with vehicle control. Akt kinase activation (P-Akt) was determined by Western blot analysis with specific antibodies to activated (phosphorylated) Akt. Samples were analyzed for total expression of Akt (data not shown) as well as for Hsc73 (loading control). TNF- α (100 ng/ml) was used as a positive control to activate Akt. Akt was inhibited by pretreatment with LY-294002 (50 μ M) for 1 h before LGG-CM or TNF treatment. Image shown is representative of 3 separate experiments.

loss of tight barrier function, and eventual cell death (11, 32, 33). Studies (32, 33) have shown previously that inducible Hsps provide cytoprotection against the oxidant stress caused by NH_2Cl in gut epithelial cells.

Pretreatment of epithelial cells with LGG-CM provides mild but statistically significant protection against oxidant damage by improving epithelial cell viability in the face of oxidant injury from NH_2Cl , as demonstrated by chromium release assay (Fig. 6A). This was further explored with a filamentous (F) to globular (G) actin assay, another functional readout of the ability of LGG-CM treatment to protect epithelial cells against oxidant stress which more specifically assesses protection against cytoskeletal damage (Fig. 6D). In the untreated controls (C), there is more F than G actin, and pretreatment

with LGG alone does not alter this ratio. Treatment of cells with NH_2Cl causes a shift from the F to the G form of actin as it disrupts the integrity of the actin cytoskeleton. Treatment with LGG before NH_2Cl results in preservation of F-actin and partial protection against NH_2Cl -induced damage to the actin cytoskeleton (last 2 lanes; compare with NH_2Cl -treated lanes).

To test the hypothesis that MAPKs, specifically p38 and JNK, play a physiologically relevant role in the mechanism of LGG-CM-mediated cytoprotection, cells were treated with inhibitors to the MAPKs after exposure to LGG-CM and the oxidant injury/chromium release assay was repeated. Consistent with this hypothesis, less cytoprotection was observed in those cells treated with the MAPK inhibitors against p38 and JNK compared with untreated (uninhibited) controls, whereas

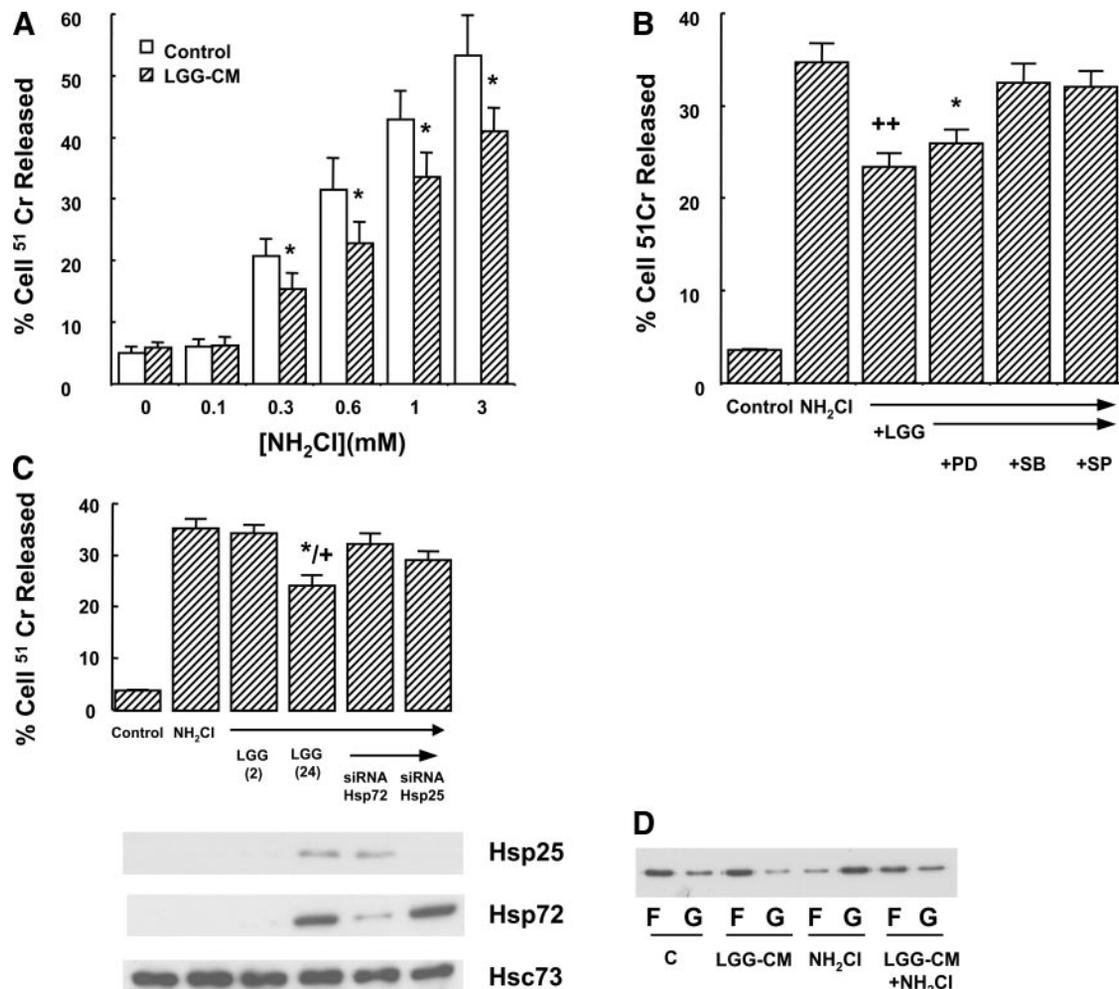


Fig. 6. LGG-CM protects YAMC cells from monochloramine (NH_2Cl) injury and oxidant-induced actin depolymerization. **A**: YAMC cells were treated with MRS (Control) or treated with 1:10 dilution of LGG-CM for 1 h, followed by removal and recovery for 15 h. Cells were labeled with ^{51}Cr for 60 min, and NH_2Cl -induced release of Cr was measured, as described in MATERIALS AND METHODS. Concentrations of NH_2Cl used are as indicated. Data are means \pm SE for 3 separate experiments; in each experiment, each point was determined in duplicate. * $P < 0.05$ compared with control by ANOVA. **B**: cells were treated with PD-98059 (50 μM), SB-203580 (20 μM), or SP-600125 (20 μM) for 2 h before addition of LGG-CM. Cells were then treated with LGG-CM for 1 h, after which media were replaced with fresh media (i.e., no inhibitors or LGG-CM) and cells were returned to the incubator overnight before chromium loading and injury with 0.6 mM NH_2Cl as described in MATERIALS AND METHODS. Data are means \pm SE for 4 separate experiments; in each experiment, each point was determined in duplicate. ++ $P < 0.001$ and * $P < 0.05$ compared with NH_2Cl -treated control by ANOVA using a Bonferroni correction. **C**: small interfering RNA (siRNA) was used to knock down Hsp25 and Hsp72 expression as described in MATERIALS AND METHODS, and cells were then treated with LGG-CM for 1 h the day before chromium loading and injury with 0.6 mM NH_2Cl as above. Data are means \pm SE for 4 separate experiments; in each experiment, each point was determined in duplicate. * $P < 0.01$ compared with NH_2Cl -treated control and + $P < 0.05$ compared with Hsp72 siRNA by ANOVA using the Bonferroni method. Western blot analysis was performed to confirm silencing of Hsp expression, and this is also shown. **D**: YAMC cells were untreated or treated with LGG-CM as above and stimulated with 0.6 mM NH_2Cl for 60 min, and cells were harvested for actin distribution in filamentous (F) and globular (G) pools as described in MATERIALS AND METHODS. Images shown are representative of 3 separate experiments.

no differences were observed in cells treated with the ERK inhibitor (Fig. 6B).

To further determine whether Hsp induction was playing a cardinal role in the cytoprotective effects of LGG-CM against oxidant stress and to assess their relative contributions to the cytoprotective effect, siRNA was used to knock down the expression of both Hsp72 and Hsp25 and the chromium release assays were repeated. Western blot analysis was performed to ensure that decreased Hsp expression had been achieved in each case (Fig. 6C). When Hsp72 expression was abolished, most of the protective effect of LGG-CM was lost (Fig. 6C). Hence, the effect of silencing Hsp expression demonstrates that, although Hsp25 may still play a role in the protection afforded by LGG-CM to oxidant injury, Hsp72 plays a greater cytoprotective role than Hsp25 against oxidant stress.

Characterization of Hsp-inducing Factor(s) in LGG-CM

After it was established that LGG-CM contained bioactive factor(s) which potently induced expression of several genes in intestinal epithelial cells, attempts were made to characterize the bioactive factors produced by these bacteria using induction of Hsp as a readout. The LGG-CM was subjected to selective ultrafiltration to determine the molecular weight of

the active factor. The filtrate (containing molecules of <10 kDa) and the retentate (containing molecules >10 kDa) or both together were then used to treat YAMC cells, and immunoblots for Hsp25 and Hsp72 were performed. Only the filtrate (lane 3) or both fractions administered together (lane 4, R+F) induced Hsp expression in YAMC cells, indicating that the bioactive factor(s) is a protein or peptide of low molecular mass <10 kDa. Further characterization of the active peptide revealed that it is heat stable, still retaining activity even after being boiled for 10 min (Fig. 7C; compare lanes 2 and 3).

As the LGG-CM bioactivity is located in the small molecular weight fraction and probiotic-derived DNA exerting bioactive and anti-inflammatory effects on gut epithelial cells has been reported (15), this possibility was explored. DNA was isolated from LGG bacteria, and epithelial cells were treated with LGG DNA over a range of several concentrations and then screened for induction of Hsp72 expression by ELISA (Fig. 7D). Compared with untreated control (see first bar, marked 0 ng/ml), no induction was seen over any of the concentrations tested, suggesting that the bioactive factor is not comprised of DNA.

To test the possibility that the bioactive factor may be a protein or peptide, LGG-CM was treated with several dif-

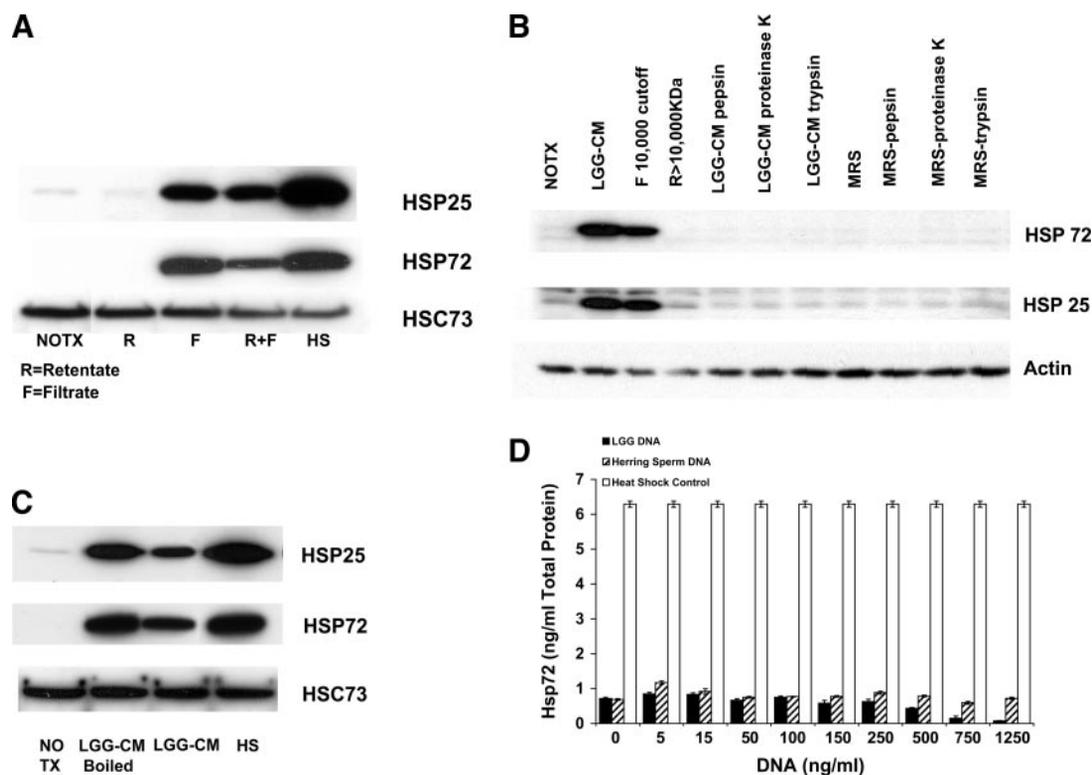


Fig. 7. Characterization of LGG-CM active factor. **A**: molecular weight determination. YAMC cells were NoTx or were treated with the retentate, filtrate, or a combination of both (R+F) from a 10-kDa molecular mass cutoff filter. Heat shock (HS) at 42°C for 23 min followed by 2-h recovery was used as a positive control. Image shown is representative of 3 separate experiments. **B**: YAMC cells were treated with vehicle control (MRS), treated with 1:10 dilution of LGG-CM alone or filtered (F), or treated with various proteases and then filtered to remove residual proteases before administration to YAMC cells. Proteases used to treat LGG-CM or MRS broth control (pepsin, trypsin, and proteinase K) are as indicated (final concentration, 50 μ g/ml each). Hsp induction was determined by Western blot analysis as described in MATERIALS AND METHODS. B-actin serves as a loading control. Images shown are representative of 3 separate experiments. **C**: heat stability. YAMC cells were untreated or treated with LGG-CM untreated (LGG) or boiled (LGG boiled), and Hsp induction was determined by Western blot analysis. Heat shock (HS) at 42°C for 23 min followed by 2-h recovery was used as a positive control. Image shown is representative of 3 separate experiments. **D**: YAMC cells were either mock treated (first bar; 0 ng/ml) or treated with DNA isolated from LGG bacteria at varying concentrations and then tested for Hsp induction using an Hsp72 ELISA. Herring sperm DNA was used as a negative DNA control, and positive control from heat shock (HS) is also shown ($n = 3$).

ferent proteases and then tested for bioactivity. First, the protease pepsin was chosen because LGG-CM has a pH of ~ 4 and pepsin is the protease with best activity at this pH. LGG was first treated with pepsin and then filtered through a 10-kDa sizing column to remove any residual pepsin. Pepsin treatment destroyed the bioactivity of LGG-CM and no induction of Hsps was seen in the YAMC cells, indicating that the bioactive factor was a protein or peptide (Fig. 7A). To confirm these findings, LGG-CM was treated with two other proteases (trypsin, proteinase K), and the proteases were then filtered out as described above. Again, Hsp-inducing activities were abolished after protease treatment, providing strong supportive evidence that the bioactive factor found in LGG-CM is a peptide or protein.

Finally, the pH of the LGG-CM was altered and then immediately used to treat the intestinal epithelial cells to determine the stability of LGG-CM at varying pH (Fig. 8A). LGG-CM is naturally at an acidic pH of 4.0 before administration to the YAMC cells (lane 3). At a neutral pH of 7.0, activity of the LGG-CM was abolished (lane 4), but if the pH was brought back to pH 4.0 and allowed to equilibrate overnight (Fig. 8B), it was possible to reestablish its Hsp72-inducing ability. (Compare the absence of Hsp72 induction in lane 7 in Fig. 8A with the Hsp72 induction in lane 4 in Fig. 8B, which is almost equivalent in intensity to the heat shock positive control.) This indicates that the peptide appears to be inactive at pH 7.0, but this is not a consequence of irreversible denaturation of the peptide, because

returning the LGG-CM to pH 4.0 for 16 h results in at least partial restoration of the bioactivity.

DISCUSSION

Probiotics may have multiple beneficial effects on the host. Soluble factors produced by a common probiotic, *Lactobacillus GG*, act on epithelial cells to produce a time- and concentration-dependent induction of the cytoprotective heat shock proteins Hsp25 and Hsp72. The soluble factors produced by LGG are sufficient for Hsp induction, and live bacteria are not required. Although conceivable that some LGG-CM-soluble factors could remain bound to cellular receptors after the cells have been thoroughly washed and all LGG-CM removed, washout experiments in which cells were exposed to LGG-CM for only a few minutes suggested that the time required to initiate the signal to the epithelial cells for upregulation of Hsps is short. Hence, although the actual appearance of Hsp protein takes hours, signal transduction pathways stimulated early appear to play a role in transmitting the instigating signal from LGG-CM to the epithelial cell.

Many protein kinases are known to be activated during the stress response, and we were able to confirm that LGG-CM exposure activates a number of MAPKs. Although there is a baseline level of activated ERK1/2 in our YAMC cells, LGG-CM pretreatment activates ERK1/2 as effectively as the phorbol ester PMA and also activates p38 and JNK. Treatment of cells with inhibitors to p38 and to JNK before LGG-CM exposure inhibits Hsp72 induction by LGG-CM with no effect on Hsc73 expression. This suggests that these two MAPKs play key roles in transmitting the cellular signal required to initiate the expression of inducible Hsps triggered by exposure to LGG-CM. It has previously been shown that *Escherichia coli* LPS induces Hsp25 through MAPK activation in YAMC cells (19), but significant differences between that study and the current one are that 1) *Lactobacillus GG* is a gram-positive organism and therefore contains no LPS, and 2) *E. coli* LPS does not induce Hsp72, whereas in this study, we have shown that Hsp72 is induced by LGG-CM through a MAPK-dependent pathway in YAMC cells in addition to Hsp25.

The reason that the ERK1/2 inhibitor has less effect on Hsp72 induction by LGG-CM is less clear, although one could argue that the presence of baseline activated ERK1/2, which we found in our cells, may provide a partial explanation. Others (26) who studied stress responses in other systems found that both p38 and JNK act through a common pathway, which is distinct from ERK1/2. The data presented herein clearly demonstrate that LGG-CM affects MAPK signaling in epithelial cells and are strongly suggestive that LGG-CM mediates its effects on Hsp production through MAPK activation. In addition, data from the chromium release assays demonstrate that the protective effects against oxidative damage conferred by LGG-CM are abolished by inhibitors to p38 and JNK, whereas ERK inhibitors have no effect. On the basis of these MAPK inhibitor data, it appears likely that rapid signaling for the induction of Hsp production is initiated through some type of signal transduction pathway involving p38 and JNK.

Our studies differ from a previous report (53) demonstrating different modulation of MAPK activities by LGG. Yan and Polk (53) reported that p38 was inhibited by live LGG bacteria,

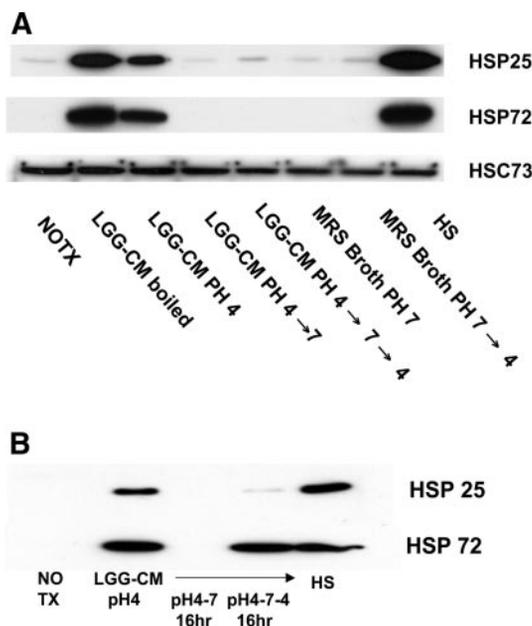


Fig. 8. The bioactive factor(s) in LGG-CM possess unusual pH properties. A: LGG-CM with different pH treatments (as indicated) was used to treat YAMC cells immediately after pH manipulation and Hsp induction was determined by Western blot analysis, as described in MATERIALS AND METHODS. MRS broth controls were subjected to the same pH treatments to ensure that the pH change itself was not causing the induction of Hsps (lanes 6 and 7). Hsc73 serves as a loading control. B: LGG-CM was treated as in A, except that after its pH level was returned to pH 4, it was left to incubate overnight (lane 4) and then used to treat YAMC cells as in A to determine whether the Hsp-inducing activity of LGG-CM could be restored over time at pH 4. Images shown are representative of 3 separate experiments.

and no effect was seen on any of the MAPKs with conditioned media alone. However, it should be noted that their LGG-CM was prepared differently: LGG cultures were grown in MRS broth as in this study, but the bacteria were then pelleted, rinsed, and resuspended in tissue culture media and allowed to grow for 2 h before CM was harvested. Our CM represents the secreted factors from a longer, overnight culture. It is possible that the bioactive factors secreted by LGG in our studies were produced only when bacteria reached a certain density and longer times are required for the bioactive factors to accumulate to a high enough concentration in the CM to display any biological effect. Indeed, we noted that it took at least 8 h in MRS broth for the LGG to secrete these factors, and the bioactive factors were not produced when grown in RPMI tissue culture media (data not shown). These differences in technique likely account for many of the differences in MAPK activation observed between the two studies.

Akt is a serine/threonine kinase which plays a pivotal role in cellular proliferation and cell survival. Akt is activated in response to several stimuli, such as growth factors, and it has been shown to play a role in the regulation of nutrient metabolism (8). LGG-CM also activates Akt, as has been shown by others (53), in intestinal epithelial cells, and as with MAPK activation, this effect is relatively rapid. It is interesting to note that binding of Hsp27 to Akt in COS cells after oxidative stress has been described (20), and one study (35) has reported that the Akt-Hsp27 binding interaction is required for Akt activation in neutrophils. Whether the same holds true in the gut epithelial cell remains to be seen.

Real-time PCR data and electrophoretic mobility shift assay analysis demonstrate that upregulation of epithelial cell Hsps is at least in part transcriptional in nature, further confirmed by microarray analysis showing that Hsps (particularly Hsp72) are among the most highly upregulated epithelial cell genes in response to LGG-CM exposure. Transient exposure to LGG-CM results in increased Hsp expression by a mechanism that at least in part is transcriptional in nature and involves the transcription factor heat shock factor-1 (HSF-1), because even short LGG-CM exposure times of only 5 min result in activation of HSF-1. It is interesting to note that the effects of LGG-CM on Hsp25 mRNA appear quite modest compared with the large relative induction of Hsp25 protein. The apparent discrepancy between amount of mRNA induction and level of protein induction seen for Hsp25 suggests that there may be posttranscriptional mechanisms of regulation involved, as has been described for other genes such as Cox-2 (7).

The cytoprotective effects of Hsps have been well described (5, 25, 32, 33, 39, 48). By inducing the expression of Hsps, bioactive factors produced by the probiotic LGG may provide additional fortification of the epithelial cell barrier and bolster intestinal defenses against hostile insults. The siRNA knockout experiments provide perhaps the most compelling evidence to this effect, showing that LGG-CM-induced Hsp72 expression provides the major cytoprotective effect against oxidative damage, with Hsp25 playing only a lesser role. Hence, the soluble products from LGG conditioned media may play a pivotal role in the ability of this probiotic to protect intestinal epithelial cells against damage from infections and inflammation. Hsp72 is known to stabilize and prevent denaturation of cellular proteins and has been shown to protect against oxidant injury in other intestinal epithelial cell lines (33). Hsp25 is an actin-

stabilizing agent, binds to actin filaments, and stabilizes filamentous actin, thus preserving cytoskeletal and tight junction functions through cytoskeletal stabilization (31). The chromium release and F-to-G-actin functional studies demonstrating cytoprotection against oxidant injury in LGG-CM-treated epithelial cells are consistent with a Hsp-mediated mechanism, although it should be noted that only partial protection is provided and other mechanisms of cytoprotection are possible (53).

LGG has been shown to decrease the duration of diarrhea caused by the pathogen rotavirus and to decrease antibiotic-associated diarrhea arising from alterations in the normal commensal flora (28, 46, 49). Rotavirus infection requires an initial interaction of the VP4 spike protein with the surface of the epithelial cell and then the COOH terminal fragment; VP5* is thought to be responsible for membrane permeabilization of the cell, which is necessary for viral entry (54). Because one role of Hsp25 is stabilization of the actin cytoskeleton, it is tempting to speculate that one possible mechanism of action accounting for LGG's beneficial effects in the setting of rotavirus infection may involve stabilization of the cellular architecture, which may render the epithelial cells more resistant to invasion by rotavirus.

The factor present in LGG-CM responsible for Hsp induction is a small molecular weight peptide, which is surprisingly acid and heat stable. These properties may provide the resilience needed for such secreted factors to survive the hostile environment of the gut during their transit through the gastrointestinal tract. It is also interesting to note that the physicochemical environment in the middle of the gut lumen is quite different from that found at the epithelial surface, which tends to be more acidic (37). This acidic microclimate has been described by several groups (30, 37) and is thought to play an important role in functions such as membrane transport, drug uptake, and nutrient absorption (42). It has been shown that the acid microclimate has a direct effect on the transport of certain dipeptides into the intestinal epithelial cell (24). If the bioactive peptides in LGG-CM act through a receptor-mediated pathway, their unusual acid-stable properties may play an important role in their ability to bind to receptors on the surface of the epithelial cell and initiate induction of cytoprotective Hsps. Efforts are currently underway to purify the bioactive peptides produced by LGG in an attempt to address some of these possibilities. Another interesting observation is that the LGG-CM is active only within a narrow concentration range, suggesting that the bioactive factors present in LGG-CM may possibly exhibit steep dose-response curves and a narrow therapeutic range of activity as has been described for some commonly used pharmacologic drugs (52), which will render this task all the more challenging.

In addition to helping to define their mechanisms of action and to explore in more depth the microbial-epithelial cell interactions which occur in the gut, there are yet other compelling reasons to pursue efforts to isolate and purify the active factor(s) in the conditioned media from the probiotic *Lactobacillus GG*. Probiotics carry a good safety record, and no increase in *Lactobacillus* bacteremia has been noted with their increasing use in a Scandinavian study from 1990 to 2000 (40). However, this study acknowledges that the risk may be higher in immunocompromised individuals, and some have raised concerns about the safety of administering live bacteria to

severely debilitated or immunocompromised patients (4, 44). Indeed, cases of *Lactobacillus* bacteremia in transplant patients have been described (2), although no attempt was made in these reports to correlate the bacteremia with probiotic use. One report in the literature describes a liver abscess in a diabetic woman taking probiotics (36). The strain of *Lactobacillus* isolated from the liver abscess was indistinguishable from the *Lactobacillus GG* strain isolated from the probiotic mixture, as determined by pulsed field gel electrophoresis analysis.

Given the wealth of knowledge now accumulating from results of both bench research and clinical trials, it is clear that probiotics do confer some benefit under specific circumstances and in certain disease states. However, the lack of regulation and quality control of probiotics is a real and recognized problem in the field (13, 47). Isolation of the beneficial bioactive factors from probiotic mixtures would eliminate the need to use live bacteria and would provide a distinct safety advantage over the use of live bacteria. In addition, one would expect that efforts aimed at isolating probiotic bioactive factors would ultimately culminate in the development of novel therapeutic agents, which could in turn be administered in a consistent and pharmacologically sound manner.

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DISCLOSURES

E. O. Petrof, J. C. Alverdy, and E. B. Chang are founders of a biotechnology company, Midway Pharmaceuticals, Inc., whose focus is to accelerate development of the probiotic bioactive factors into therapeutic agents.

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