Cellular Microbiology (2008) **10**(7), 1442–1452
doi:10.1111/j.1462-5822.2008.01137.x
First published online 26 March 2008

Probiotic *Lactobacillus reuteri* promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-κB and MAPK signalling

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Summary

The molecular mechanisms of pro-apoptotic effects of human-derived *Lactobacillus reuteri* ATCC PTA 6475 were investigated in this study. *L. reuteri* secretes factors that potentiate apoptosis in myeloid leukemia-derived cells induced by tumour necrosis factor (TNF), as indicated by intracellular esterase activity, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling assays and poly (ADP-ribose) polymerase cleavage. *L. reuteri* downregulated nuclear factor-κB (NF-κB)-dependent gene products that mediate cell proliferation (Cox-2, cyclin D1) and cell survival (Bcl-2, Bcl-xL). *L. reuteri* suppressed TNF-induced NF-κB activation, including NF-κB-dependent reporter gene expression in a dose-and time-dependent manner. *L. reuteri* stabilized degradation of IκBα and inhibited nuclear translocation of p65 (RelA). Although phosphorylation of IκBα was not affected, subsequent polyubiquitination necessary for regulated IκBα degradation was abrogated by *L. reuteri*. In addition, *L. reuteri* promoted apoptosis by enhancing mitogen-activated protein kinase (MAPK) activities including c-Jun N-terminal kinase and p38 MAPK. In contrast, *L. reuteri* suppressed extracellular signal-regulated kinases 1/2 in TNF-activated myeloid cells. *L. reuteri* may regulate cell proliferation by promoting apoptosis of activated immune cells via inhibition of IκBα ubiquitination and enhancing pro-apoptotic MAPK signalling. An improved understanding of *L. reuteri*-mediated effects on apoptotic signalling pathways may facilitate development of future probiotics-based regimens for prevention of colorectal cancer and inflammatory bowel disease.

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Introduction

Lactobacillus and *Bifidobacterium* spp. represent sources of beneficial organisms termed probiotics, which are defined as 'live microorganisms which when administered in adequate amounts confer health benefits to the host' (FAO/WHO, 2001). Therapeutic effects of probiotics include alleviation of symptoms of lactose malabsorption, enhanced natural resistance to infectious diseases and chronic inflammation of the gastrointestinal tract, reduction in serum cholesterol concentration, stimulation of gastrointestinal immunity and suppression of colon cancer (Gilliland *et al.*, 1985; Salminen *et al.*, 1998; Perdigon *et al.*, 2001; Rafter, 2003; Isolauri *et al.*, 2004; Guarner *et al.*, 2006). Probiotics are attractive candidates for novel biological therapies because beneficial bacteria may be derived from commensal microorganisms and are generally recognized as safe microbes.

Probiotic bacteria can modulate systemic inflammation, cell proliferation and apoptosis, and such properties may be useful for future immunomodulatory and cancer prevention strategies (Kato *et al.*, 1998; Sheil *et al.*, 2004). Anti-proliferative and pro-apoptotic effects of *Lactobacillus* and *Bifidobacterium* spp. on various cancer cell lines have been demonstrated (Fichera and Giese, 1994; Biffi *et al.*, 1997; Kim *et al.*, 2002). Reports also indicated that probiotic strains inhibited liver, bladder and mammary tumours in animal models, highlighting potentially systemic effects of probiotics with anti-neoplastic activities (Reddy and Rivenson, 1993; Lim *et al.*, 2002; de Moreno de Leblanc *et al.*, 2007). In double-blinded studies of patients fed *Lactobacillus casei* Shirota preparations, Aso *et al.* (1995) reported the suppression of bladder tumour recurrence. *Lactobacillus reuteri* represents a

commensal-derived probiotic species with potent anti-inflammatory and anti-proliferative effects (Ma *et al*., 2004; Pena *et al*., 2005; Smits *et al*., 2005). *L. reuteri* is indigenous to humans and widely prevalent in animals (Reuter, 2001). Ma *et al.* (2004) reported that *L. reuteri* mediated its anti-inflammatory effects via inhibition of nuclear translocation of nuclear factor-κB (NF-κB) signalling in human intestinal epithelial cells. However, the precise mechanism by which *L. reuteri* modulates cell proliferation and apoptosis remains unknown.

Promotion of apoptosis in neoplastic cells is highly desirable as a cancer prevention strategy. Apoptosis is regulated by intracellular signalling pathways that include key factors such as NF-κB and mitogen-activated protein kinases (MAPKs) (Aggarwal, 2003). Because of the central role of NF-κB and MAPK signalling in inflammation, cell proliferation and apoptosis, we speculated that *L. reuteri* mediated anti-proliferative and pro-apoptotic effects by modulating NF-κB and MAPK signalling pathways. This report, which describes anti-proliferative and pro-apoptotic activities via multiple signalling pathways, represents a novel paradigm for probiotics and therapeutic microbiology.

Results

This study investigated the effects of *L. reuteri* ATCC 6475 supernatant (Lr-S 6475) on tumour necrosis factor (TNF)-activated apoptosis signalling pathways in myeloid leukemia-derived cells. These studies were performed using human chronic myeloid leukemia-derived cells (KBM-5) because these cells are considered to be a well-established system for NF-κB and MAPK signalling (Ichikawa *et al*., 2006; Ahn *et al*., 2007) and express sufficient quantities of TNF receptors 1 and 2 in their baseline state.

Lactobacillus reuteri *promoted TNF-induced apoptosis*

Several methods were utilized to explore cell necrosis and apoptosis including intracellular esterase activity and plasma membrane integrity. Probiotic Lr-S 6475 enhanced TNF-induced cytotoxicity from 3% to 38% (Fig. 1A), as determined by fluorophore staining. In the absence of TNF, probiotic treatment of cells for 24 h did not diminish cell viability as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Fig. 1B) and trypan blue methods (data not shown). However, MTT assays confirmed the cytotoxic effects of Lr-S 6475 on myeloid cells in the presence of TNF (Fig. 1B). Probiotic-mediated enhancement of TNF-induced apoptosis was investigated by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) assays, which detect discontinuities in DNA strands as a sign of apoptosis. TNF alone was minimally effective in inducing apoptosis; however, when Lr-S 6475 and TNF were used together, the proportions of apoptotic KBM-5 cells increased from 3% to 37% as determined by TUNEL (Fig. 1C), indicating that probiotic *L. reuteri* 6475 upregulated TNF-induced apoptosis.

Immunoblot analyses of extracts from cells treated with Lr-S 6475 and TNF yielded evidence of activation of downstream caspases, as indicated by poly (ADP-ribose) polymerase (PARP) cleavage. In KBM-5 cells pre-treated with Lr-S 6475, the 116 kDa PARP protein was cleaved to yield a 85 kDa fragment, a hallmark of cells undergoing apoptosis (Fig. 1D). In the absence of secreted factors of probiotic *L. reuteri*, KBM-5 cells did not show visible evidence of PARP cleavage in response to TNF treatment. The combined results from this study showed that Lr-S 6475 enhanced TNF-induced apoptosis in human myeloid leukemia-derived cells.

Lactobacillus reuteri *suppressed cell proliferation and anti-apoptotic proteins*

Cyclin D1 and Cox-2 promote cell proliferation and may contribute to carcinogenesis. Cyclin D1 controls the G_1-S transition and is highly expressed in a variety of tumours (Alao, 2007). Cox-2 is an enzyme that catalyses the production of PGE_2 from arachidonic acid. The production of PGE_2 has been linked to proliferation and metastasis of tumour cells (Jachak, 2007). TNF treatment induced the levels of Cox-2 and cyclin D1 proteins in human myeloid cells, whereas pre-treatment of cells with Lr-S 6475 suppressed TNF-induced Cox-2 and cyclin D1 proteins (Fig. 2).

The anti-apoptotic proteins, Bcl-2 and Bcl-xL, suppress apoptosis and prolong survival of neoplastic cells (Adams and Cory, 2007). Immunoblot analyses showed that TNF induced the expression of Bcl-2 and Bcl-xL in a time-dependent manner, and Lr-S 6475 pre-treatment significantly suppressed production of Bcl-2 and Bcl-xL in the presence of TNF (Fig. 2). Lr-S 6475 had no effect on protein synthesis in general as indicated by the unchanged expression of β-actin (Fig. 2). Probiotic Lr-S 6475 may promote apoptosis via suppression of proteins involved in cell proliferation and anti-apoptosis.

Lactobacillus reuteri *inhibited TNF-dependent NF-κB activation*

Because NF-κB signalling is an important regulatory pathway for cell proliferation and apoptosis, the effects of Lr-S 6475 on TNF-induced NF-κB activation were investigated. Cells were treated with Lr-S 6475 (20% v/v) for 16 h and exposed to TNF (0.1 nM) for different time intervals.

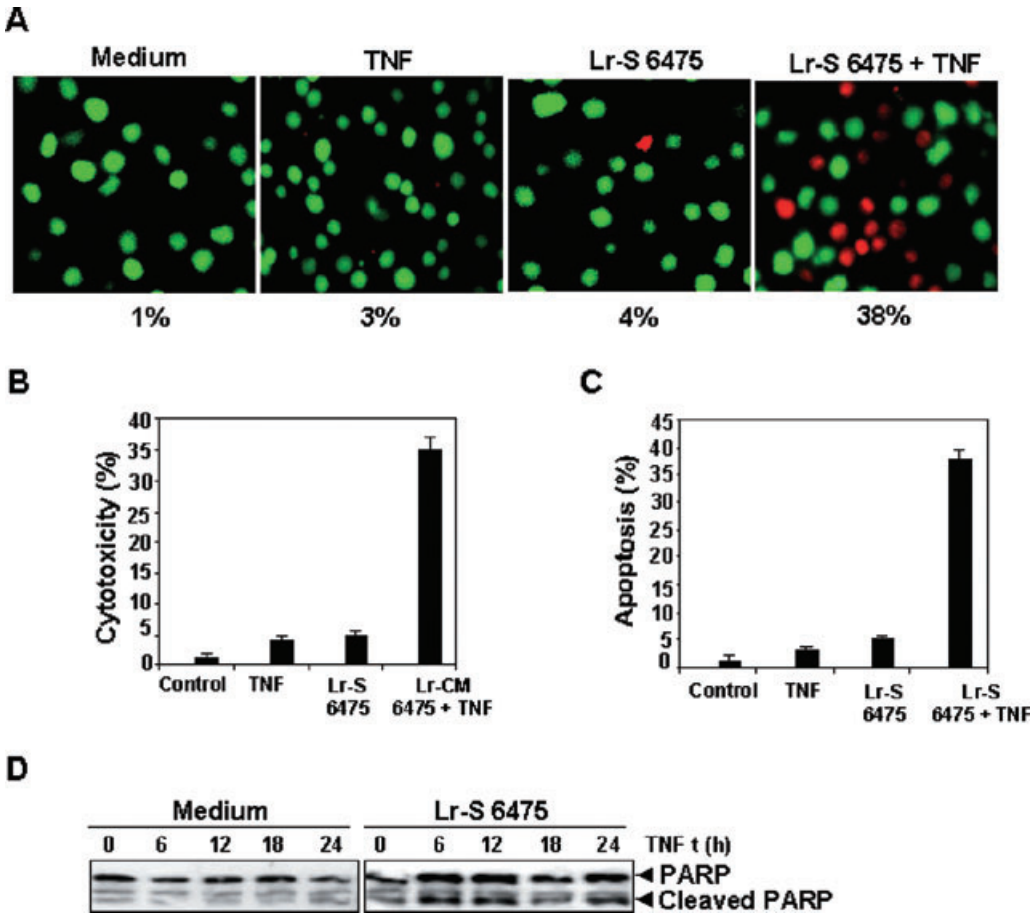


Fig. 1. *L. reuteri* enhanced TNF-induced apoptosis in myeloid leukemia-derived cells.
A. KBM-5 cells (1×10^6 cells ml^{-1}) were incubated with TNF, alone or in combination with Lr-S 6475 (20% v/v), as indicated for 24 h. Cell death was determined by the calcein-AM-based live/dead assay as described under *Experimental procedures*. Red colour highlights dead cells, and green colour highlights viable cells.
B. KBM-5 cells (5000 cells per well) were incubated at 37°C with TNF (1 nM), in the presence and absence of Lr-S 6475 (20% v/v) for 24 h, and the viable cells were assayed using the MTT reagent. The results are shown as the mean ± SD from triplicate cultures.
C. KBM-5 cells were pre-treated with Lr-S 6475 (20% v/v) and TNF (1 nM) for 24 h. Cells were fixed, stained with TUNEL assay reagent, and analysed by fluorescence microscopy.
D. Cells were pre-treated with Lr-S 6475 (20% v/v) for 16 h and incubated with TNF (1 nM) for the indicated times. Whole-cell extracts were prepared and subjected to immunoblot analyses using anti-PARP antibody in order to detect PARP (uncleaved) and cleaved PARP. Cleaved PARP indicates promotion of apoptosis. t (h) refers to time in hours.

Nuclear extracts were prepared and examined for evidence of NF-κB activation by DNA-binding assays. TNF activated NF-κB in a time-dependent manner in the untreated cells (Fig. 3A, left panel); however, in Lr-S 6475-treated cells, NF-κB activation was significantly inhibited (Fig. 3A, right panel). To determine the optimum time of Lr-S 6475 exposure in order to suppress NF-κB activation, cells were treated with Lr-S 6475 (20% v/v) for different time intervals prior to activation with TNF (0.1 nM). Lr-S 6475 alone did not activate NF-κB (Fig. 3B, left panel), but TNF-induced NF-κB activation was inhibited by Lr-S 6475 in a time-dependent manner (Fig. 3B, right panel). To determine the optimum dose of Lr-S 6475 required to suppress NF-κB activation, cells were treated with different concentrations of Lr-S 6475 for 16 h prior to activation with TNF (0.1 nM). Lr-S 6475 did not activate NF-κB (Fig. 3C, left panel), but pre-treatment of human myeloid cells with Lr-S 6475 inhibited TNF-induced NF-κB activation in a dose-dependent manner (Fig. 3C, right panel).

Nuclear factor-κB is composed of combinations of Rel/NF-κB. Active NF-κB heterodimers bind specific DNA sequences (Karin and Greten, 2005). When nuclear extracts from TNF-activated cells were incubated with antibodies to p50 (NF-κB1) and p65 (RelA) subunits of NF-κB, resulting band shifts indicated that TNF-activated complexes consisted of p50 or p65 subunits. The addition of excess unlabelled NF-κB (cold oligonucleotide, 100-fold) caused a complete disappearance of the band, whereas mutated oligonucleotides did not affect DNA binding (data not shown). Nuclear extracts from TNF-

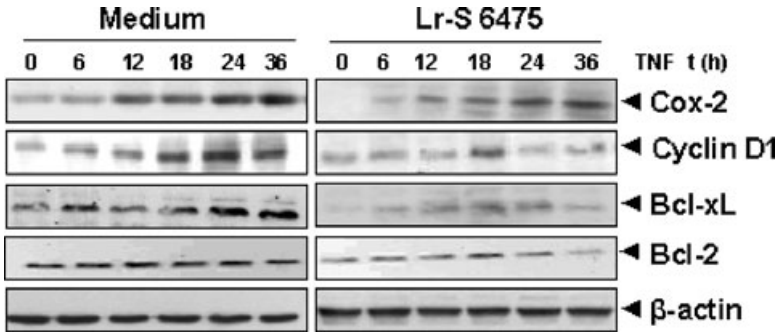


Fig. 2. *L. reuteri* suppressed cell proliferation and anti-apoptotic proteins. KBM-5 cells (2×10^6 cells ml^{-1}) were left untreated or incubated with Lr-S 6475 (20% v/v) for 16 h and treated with 1 nM TNF for different times. Whole-cell extracts were prepared, and 50 μg of each whole-cell lysate was resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, and probed with antibodies against Bcl-xL, Bcl-2, Cox-2 and cyclin D1, or β-actin. TNF-treated and TNF plus Lr-S 6475-treated samples were size-fractionated in the same gel under identical conditions. The results shown were representative of three independent experiments. t (h) refers to time in hours.

induced cells were incubated with Lr-S 6475 and analysed for DNA binding activity by electrophoretic mobility shift assay (EMSA). Lr-S 6475 did not interfere with the DNA binding ability of the NF-κB complex (data not shown).

Lactobacillus reuteri *inhibited TNF-induced NF-κB-dependent reporter gene expression*

As DNA binding may not correlate with effects on gene

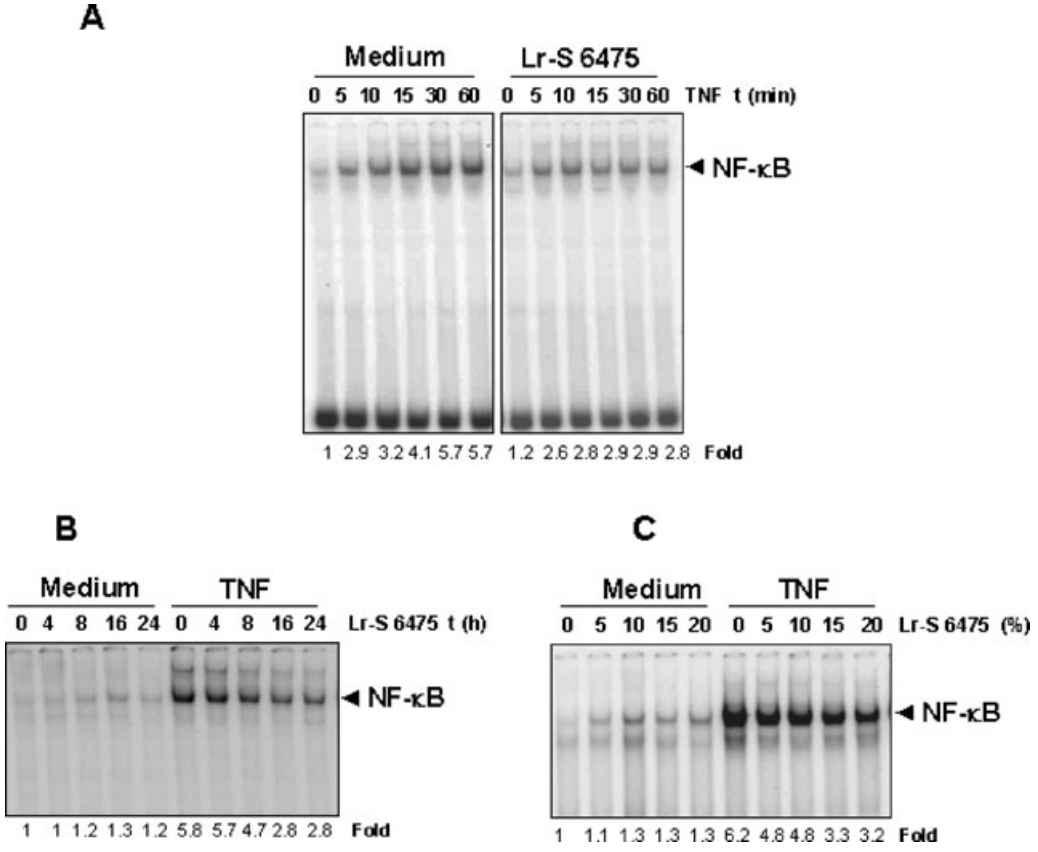


Fig. 3. *L. reuteri* inhibited TNF-dependent NF-κB activation.
A. KBM-5 cells were left untreated or incubated with Lr-S 6475 (20% v/v) for 16 h, treated with human TNF (0.1 nM) for the indicated times and analysed for evidence of NF-κB activation by EMSA.
B. Cells were incubated with Lr-S 6475 (20% v/v) for the indicated times, treated with human TNF (0.1 nM) for 30 min, and subjected to EMSA studies to probe NF-κB activation.
C. Cells were incubated with different concentrations of Lr-S 6475 for 16 h, treated with TNF (0.1 nM) for 30 min, and analysed for evidence of NF-κB activation by EMSA. t (h) refers to time in hours.

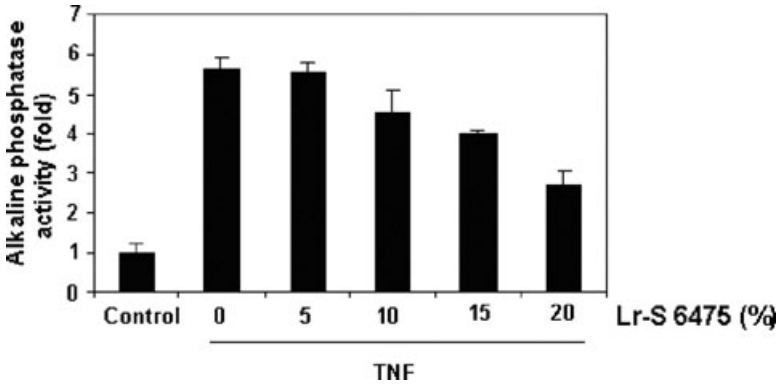


Fig. 4. *L. reuteri* repressed NF-κB-dependent reporter gene expression induced by TNF. A293 cells were transiently transfected with a plasmid-containing NF-κB binding site immediately upstream of the SEAP reporter gene. After 16 h incubation, A293 cells were incubated with different concentrations of Lr-S 6475 for 16 h and treated with TNF (1 nM) for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity (data presented as mean ± SD of three different experiments) by chemiluminescence at 570 nm.

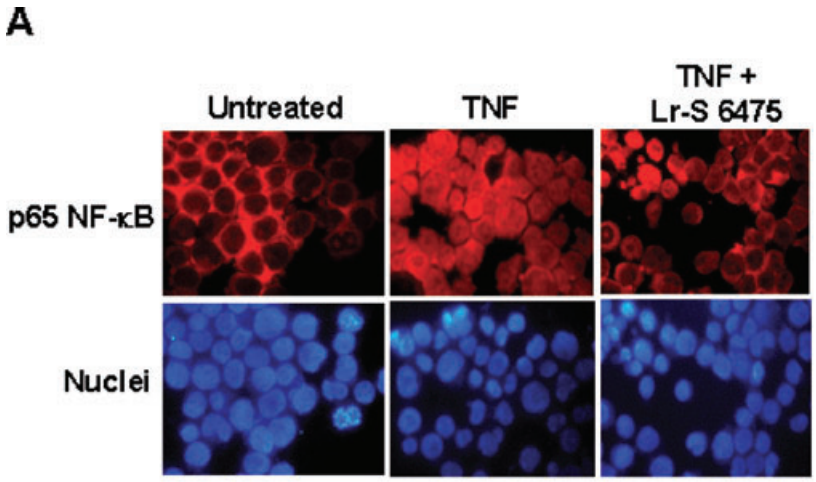
expression (Nasuhara *et al.*, 1999), effects of probiotic mediators on TNF-induced NF-κB-dependent reporter gene transcription was evaluated. The results of secretory alkaline phosphatase (SEAP) reporter assay showed that TNF induced NF-κB-regulated reporter gene expression, and Lr-S 6475 suppressed NF-κB activation in a dose-dependent manner (Fig. 4).

Lactobacillus reuteri *inhibited p65 translocation into the nucleus*

The degradation of IκBα resulted in nuclear translocation of p65. Effects of Lr-S 6475 on TNF-induced nuclear translocation were examined. Data from immunocytochemical analyses showed that TNF induced p65 nuclear translocation within 20 min in KBM-5 cells as expected, and Lr-S 6475 pre-treated cells significantly suppressed TNF-induced nuclear translocation of p65 (Fig. 5A).

Lactobacillus reuteri *inhibited IκBα degradation*

Nuclear translocation of NF-κB is preceded by proteolytic degradation of IκBα (Karin and Greten, 2005). To determine whether probiotics could suppress NF-κB activation by inhibition of IκBα degradation, KBM-5 cells were pre-treated with Lr-S 6475 and exposed to TNF (0.1 nM) at



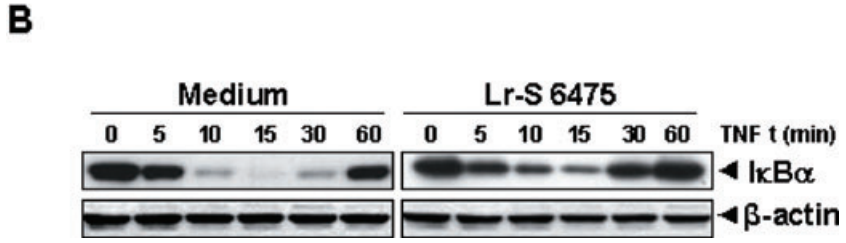


Fig. 5. *L. reuteri* inhibited TNF-induced IκBα degradation.
A. KBM-5 cells were treated with Lr-S 6475 (20% v/v) for 16 h at 37°C and exposed to TNF (1 nM) for 20 min. Immunocytochemical analyses of p65 localization was determined as described under *Experimental procedures*. The results shown are representative of three independent experiments.
B. KBM-5 cells were incubated with Lr-S 6475 (20% v/v) for 16 h and treated with human TNF (0.1 nM) for the indicated times. Cytoplasmic extracts were prepared, fractionated in 10% SDS-PAGE, and electro-transferred to nitrocellulose membranes. Immunoblot analyses were performed with anti-IκBα and detected by chemiluminescence. t (h) refers to time in hours.

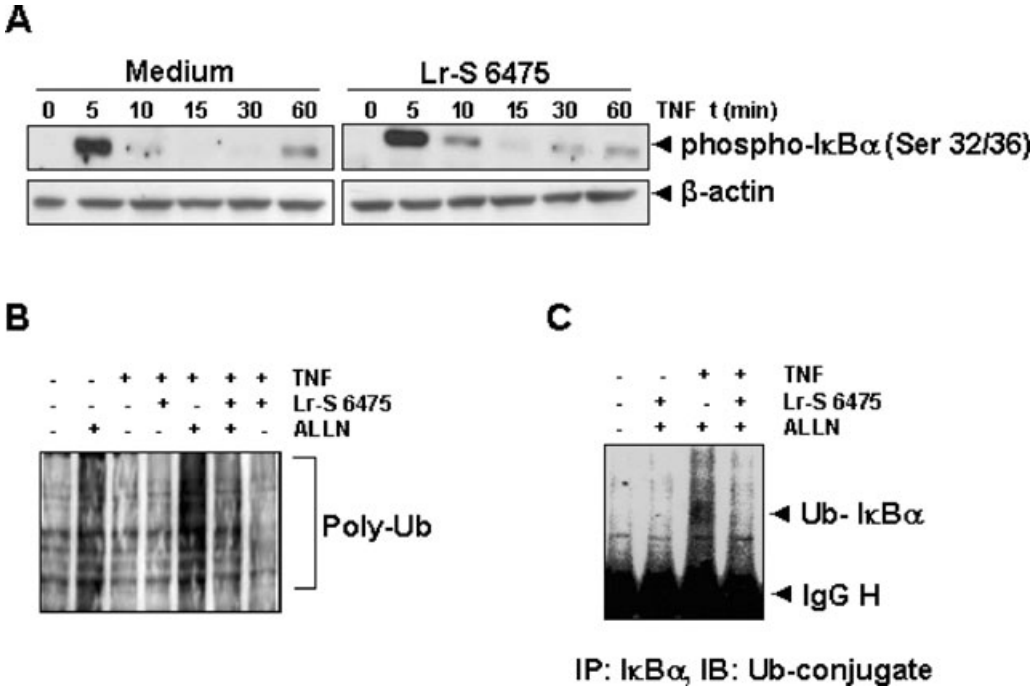


Fig. 6. *L. reuteri* inhibited TNF-induced IκBα ubiquitination.
A. KBM-5 cells were pre-incubated with Lr-S 6475 (20% v/v) for 16 h, incubated with 50 μg ml^{-1} ALLN for 30 min, and treated with TNF (0.1 nM) for indicated times. Cytoplasmic extracts were fractionated and subjected to immunoblot analyses using antibodies specific for phospho-IκBα (Ser 32/36) antibody.
B. KBM-5 cells were pre-incubated with Lr-S 6475 (20% v/v) for 16 h, incubated with ALLN (50 μg ml^{-1}) for 30 min, and treated with TNF (0.1 nM) for 15 min. Cytoplasmic extracts were fractionated and subjected to immunoblot analyses using ubiquitin-specific antibodies.
C. KBM-5 cells (2×10^6 cells ml^{-1}) were pre-incubated with Lr-S 6475 (20% v/v), incubated with ALLN (50 μg ml^{-1}) and treated with TNF (1 nM). Whole-cell extracts were prepared and incubated with anti-IκBα antibody. Immune complexes were precipitated with protein A/G-Sepharose beads and samples were subjected to SDS-PAGE and immunoblotting with anti-ubiquitin antibodies. t (h): time in hours; IgG H, human immunoglobulin G; Poly Ub, polyubiquitinated proteins; Ub-IκBα, ubiquitinated inhibitory subunit of NF-κB.

different time intervals. The IκBα status in the cytoplasm was analysed by immunoblot studies. TNF induced IκBα degradation in control cells (not treated with *L. reuteri* secreted factors) within 10 min, but Lr-S 6475 pre-treatment stabilized IκBα in the presence of TNF (Fig. 5B). These results indicate that Lr-S 6475 suppressed TNF-induced IκBα degradation.

Lactobacillus reuteri *suppressed IκBα ubiquitination*

To determine whether the inhibition of TNF-induced IκBα degradation was due to inhibition of IκBα phosphorylation, the proteasomal degradation inhibitor *N*-acetyl-leucylleucyl-norleucinal (ALLN) was used to block degradation of IκBα. KBM-5 cells were pre-treated with Lr-S 6475, prior to exposure to ALLN and TNF. The phosphorylation status of IκBα was evaluated by immunoblot analyses using an antibody that recognizes the serine-phosphorylated form of IκBα. TNF induced phosphorylation of IκBα within 5 min after binding to TNF receptors in myeloid cells (Fig. 5B, left panel). Lr-S 6475 pretreatment did not affect TNF-induced phosphorylation of IκBα in the presence of the proteasomal inhibitor (Fig. 5B, right panel). This result suggests that TNF binds effectively to its receptors despite the presence of probiotics. Probiotics may suppress phospho-IκBα degradation in response to TNF by interfering with downstream effectors such as ubiquitination or proteasomal degradation of IκBα. To explore this question, KBM-5 cells were pre-treated with Lr-S 6475, prior to exposure with ALLN and TNF. Results from immunoblot analyses and immunoprecipitation experiments showed that Lr-S 6475 inhibited both polyubiquitination and IκBα-specific ubiquitination respectively (Fig. 6B and C).

Lactobacillus reuteri *affected MAPK phosphorylation*

In view of evidence that MAPKs, such as c-Jun N-terminal kinases (JNK), p38 and extracellular signal-regulated kinases (ERK), play a critical role in cell survival and apoptosis, the effects of *L. reuteri* on MAPK signalling pathways were examined. Incubation of KBM-5 cells with TNF resulted in phosphorylation of JNK, p38 and ERK (Fig. 7). Pre-treatment with Lr-S 6475 enhanced JNK and p38 phosphorylation, but suppressed ERK1/2 signalling in TNF-treated KBM-5 cells (Fig. 7).

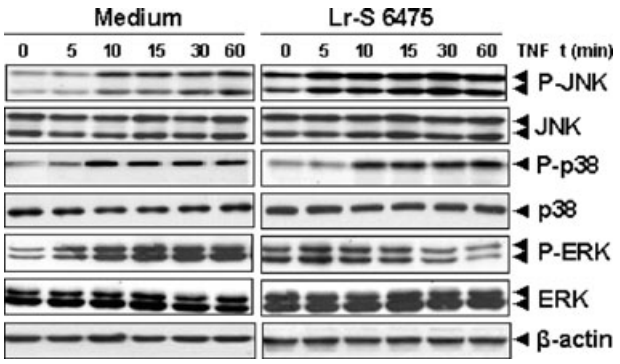


Fig. 7. *L. reuteri* modulated MAPK phosphorylation. KBM-5 cells (2×10^6 cells ml^{-1}) were left untreated or incubated with Lr-S 6475 (20% v/v) for 16 h and treated with 1 nM TNF for different times. Whole-cell extracts were prepared, and 50 μg of each whole-cell lysate was resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, and probed with antibodies against phospho-p38, p38, phospho-JNK, JNK, phospho-ERK, ERK or β-actin. TNF-treated and TNF plus Lr-S 6475-treated samples were size-fractionated in the same gel under identical conditions. The results were representative of three independent experiments. t (h) refers to time in hours.

Discussion

This report includes the first documentation that probiotics promote apoptosis in human myeloid cells via modulation of NF-κB and MAPK signalling. Results from this study showed that *L. reuteri* potentiated TNF-induced apoptosis via downregulation of NF-κB signalling and expression of gene products dependent on NF-κB activation. TNF treatment induced the expression of anti-proliferative and anti-apoptotic proteins in human myeloid cells. *L. reuteri* 6475 secrete factors (Lr-S 6475) that suppressed TNF-induced expression of anti-apoptotic (Bcl-2 and Bcl-xL) and cell-proliferative proteins (Cox-2 and cyclin D1). Suppression of TNF-induced Bcl-2 and Bcl-xL proteins by Lr-S 6475 correlated with the potentiation of apoptosis by TNF. Cox-2 has been implicated in carcinogenesis, and its overexpression in neoplastic cells enhanced cellular invasion, angiogenesis and antiapoptosis (Hirschowitz *et al.*, 2002). Effects of Lr-S 6475 on the cell cycle and cell proliferation could be mediated through the downregulation of Cox-2 and cyclin D1.

Because Cox-2, cyclin D1, Bcl-2 and Bcl-xL are regulated by NF-κB, the effects of *L. reuteri* on NF-κB signalling were investigated. Lr-S 6475 suppressed TNF-induced NF-κB activation in a dose- and time-dependent manner. Pro-inflammatory stimuli, such as TNF, activate NF-κB through a tightly regulated pathway including phosphorylation, ubiquitination and proteolytic events (Karin and Greten, 2005). *L. reuteri* did not interfere with the ability of NF-κB to bind to DNA targets (data not shown). However, probiotic *L. reuteri* secreted factors that inhibited the ubiquitination of IκBα but did not inhibit TNF-induced IκBα phosphorylation. By suppressing ubiquitination of IκBα, *L. reuteri* abrogated NF-κB signalling. Polyubiquitinated IκBα is targeted for degradation by the 26S proteasome (Chen *et al.*, 1995), facilitating the nuclear translocation of NF-κB, and sequence-specific recognition of target promoters. *Lactobacillus* spp. inhibited TNF-induced NF-κB activation in intestinal epithelial cells through a proteasomal degradation pathway (Petrof *et al.*, 2004; Tien *et al.*, 2006), suggesting that different probiotics may mediate effects on different signalling pathways. The current studies showed that the attenuation of NF-κB activation by probiotics may be mediated by secreted factors via a contact-independent mechanism.

Commensal bacteria, including *Lactobacillus* spp., influenced the regulatory pathways of the mammalian intestinal epithelium by directly modulating the ubiquitin–proteasome system (Kumar *et al.*, 2007). Direct contact of a non-pathogenic *Salmonella typhimurium* strain with intestinal epithelial cells inhibited IκBα degradation (Neish *et al.*, 2000). Another commensal bacterium *Bacteroides thetaiotaomicron* attenuated pro-inflammatory cytokine expression by inducing nuclear export of complexes formed by NF-κB and peroxisome proliferator-activated receptor-γ. The biological action of *B. thetaiotaomicron* is downstream of NF-κB activation (Kelly *et al.*, 2004), whereas other commensal bacteria (including *L. reuteri* reported in this study) blocked NF-κB signalling at more proximal steps such as stabilization of IκBα (Neish *et al.*, 2000; Kelly *et al.*, 2004; Petrof *et al.*, 2004). Suppression of NF-κB activation by probiotics in activated cells may be most effective for cancer prevention.

Apart from NF-κB signalling, MAPKs contribute to TNF-induced apoptotic signalling pathways (Lin and Dibling, 2002; Tanaka *et al.*, 2002; Efimova *et al.*, 2004; Grethe and Porn-Ares, 2006; Meloche and Pouyssegur, 2007). Results from this study showed that *L. reuteri* enhanced TNF-induced phosphorylation of JNK and p38 proteins, but suppressed ERK1/2 phosphorylation in TNF-treated KBM-5 cells. The ERK1/2 pathway regulates the activity of cyclin D1, a key protein involved in cell proliferation (Hayashi and Lee, 2004). Suppression of cyclin D1 in Lr-S 6475-treated myeloid cells may be partially due to inhibition of the ERK1/2 pathway. Several studies have validated ERK1/2 inhibition as an attractive target for cancer therapy (Dong *et al.*, 2000; Gray-Schopfer *et al.*, 2007; Roberts and Der, 2007).

Recent studies reveal that JNK activation may be negatively regulated by NF-κB-mediated inhibition (De Smaele *et al.*, 2001; Tang *et al.*, 2001). JNK promoted TNF-induced apoptosis only in the absence of NF-κB activation (Lin and Dibling, 2002; Tang *et al.*, 2002). *L. reuteri* may potentiate TNF-induced apoptosis via suppression of NF-κB activation, thereby allowing prolonged JNK activation. The p38 and JNK signalling pathways have

antagonistic effects on ERK signalling (Efimova *et al*., 2004; Friedman and Perrimon, 2006) and can serve as additional means of regulating apoptosis via enhanced p38 and JNK phosphorylation (Xia *et al*., 1995). Recent findings from our laboratory also suggest that a downstream transcription factor of JNK activation, AP-1, is regulated by probiotic *L. reuteri* in cells treated with Toll-like receptor agonists (Y. Lin, unpubl. data).

Although direct involvement of Lr-S 6475 in MAPK signalling is not shown, *L. reuteri* may mediate pro-apoptotic effects via multiple signalling pathways including NF-κB and MAPKs. Therefore, *L. reuteri* may regulate cancer cell proliferation by altering levels of key proteins participating in apoptosis and affecting the aggregate balance of pro- and anti-apoptotic factors within TNF-stimulated cells. Similarly, de Moreno de Leblanc *et al.* (2007) who demonstrated that milk fermented with *Lactobacillus helveticus* reduced Bcl-2 expression and enhanced apoptosis in a murine cancer model. In addition, *L. casei* suppressed Bcl-2 gene expression in mucosal T lymphocytes of patients with Crohn's disease (Carol *et al*., 2006). In contrast, *Lactobacillus rhamnosus* GG prevented TNF-induced apoptosis in human and mouse intestinal epithelial cells by secretion of two bacterial proteins (40 and 75 kDa) (Yan and Polk, 2002; Yan *et al*., 2007). Different probiotics may differentially regulate cellular signalling pathways and ultimately yield different effects with respect to mammalian cell cycle regulation and cell proliferation.

In summary, these results provide clues to understanding molecular mechanisms by which *L. reuteri* mediates its anti-proliferative and pro-apoptotic effects in neoplastic cells. The isolation and identification of *L. reuteri* secreted factors (studies in progress) may also provide an improved understanding of cellular signalling associated with apoptotic signalling pathways. Further animal and human studies are needed in order to realize the therapeutic potential of commensal-derived probiotics for disorders of chronic inflammation and cancer prevention.

Experimental procedures

Reagents

Recombinant human TNF was purchased from Chemicon (Temecula, CA). Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Grand Island, NY). Antibodies against NF-κB subunits (p65, p50), IkBα, cyclin D1, PARP, Bcl-2 and Bcl-xL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclooxygenase (Cox)-2 was purchased from BD Biosciences (San Jose, CA). Antibodies against phospho-p38, p38, phospho-JNK, JNK, phospho-ERK, ERK, Phospho-specific anti-IκBα (serine 32/36) and phospho-specific anti-p65 (serine 536) antibodies were purchased from Cell Signalling Technology (Beverly, MA). Anti-ubiquitin and β-actin antibodies were obtained from Sigma (St Louis, MO). ALLN was purchased from Calbiochem (San Diego, CA).

Cell lines

Human chronic myeloid leukemia-derived cells (KBM-5 cells) and human embryonic kidney cells (A293 cells) were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in IMDM supplemented with 15% fetal bovine serum. A293 cells were cultured in DMEM supplemented with 10% fetal bovine serum.

Bacterial culture and supernatant

Lactobacillus reuteri ATCC PTA 6475 (*L. reuteri* 6475) was grown in a modified defined medium named LDM III (Kotarski and Savage, 1979). Overnight cultures were diluted to an OD_{600} of 1.0 (which equates to approximately 10^9 cells ml^{-1}). Lr-S 6475 was collected by centrifugation at 4000 r.p.m. for 10 min at 4°C. The supernatants were collected by filtration through a pore size of 0.22 μm (Millipore, Bedford, MA).

Cytotoxicity assays

The effects of Lr-S 6475 on the cytotoxic effects of TNF was determined by modified tetrazolium salt MTT uptake method as previously described (Shishodia and Aggarwal, 2004). Briefly, 5000 cells (KBM-5) were incubated with Lr-S 6475 (20% v/v) in triplicate in 96-well plates at 37°C for 48 h. MTT solution was then added to each well. After a 2 h incubation at 37°C, extraction buffer (20% SDS, 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and the optical densities were measured at 570 nm using a 96-well plate reader (Dynex Technologies, MRX Revelation, Chantilly, VA, USA). Cytotoxic effects of Lr-S 6475 were further confirmed by Live and Dead assay (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic dye, which is retained in live cells and yields green fluorescence. It also uses the ethidium monomer dye (red fluorescence), which enters cells only through damaged membranes and binds to nucleic acids but is excluded by the intact plasma membrane of live cells. Briefly, 2×10^5 cells were incubated with Lr-S 6475 (20% v/v) for 16 h and then treated with TNF (1 nM) for 24 h at 37°C. Cells were stained with the Live and Dead reagent (20% ethidium homodimer and 20% calcein-AM) and incubated at 37°C for 30 min. Cells were analysed by fluorescence microscopy (Labophot-2; Nikon, Tokyo, Japan).

The TUNEL assay

Apoptosis was detected with an ApopTag *in situ* detection kit from Chemicon (Temecula, CA), which employs TUNEL methodology. Briefly, cells were harvested and centrifuged at 800 *g* for 10 min at 4°C, and the pellet was fixed in 10% formalin overnight. After quenching of endogenous peroxidase activity, samples were incubated with a TdT enzyme mix (60 min at 37°C in a humidified chamber). Incorporated digoxigenin-labelled nucleotides were detected by using an anti-digoxigenin peroxidase-conjugated

monoclonal antibody fragment. Bound peroxidase activity was detected with filtered diaminobenzidine (DAB), producing an insoluble brown-black precipitate. Slides were counterstained in hematoxylin, destained in distilled water, and dehydrated prior to mounting of coverslips with Permount. Images were examined using an Olympus BX51 microscope with an Olympus DP71 camera. The number of TUNEL-positive myeloid cells was expressed as a percentage of total DAB-positive stained cells.

poly (ADP-ribose) polymerase cleavage assay

For detection of PARP cleavage products, whole-cell extracts were prepared by subjecting Lr-S 6475-treated cells to lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.01 μg ml^{-1} aprotinin, 0.005 μg ml^{-1} leupeptin, 0.4 mM phenylmethylsulfonyl fluoride and 4 mM $NaVO_4$). Lysates were spun at 14 000 r.p.m. for 10 min to remove insoluble material, resolved by 7.5% SDS-PAGE, and probed with PARP antibodies.

Western blot analyses

KBM-5 cells were washed with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer containing 1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM Na-pyrophosphate, 1 mM Na_3VO_4, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 10 μg ml^{-1} aprotinin. Proteins were resolved by 10% SDS-PAGE, electroblotted to nitrocellulose membranes (Bio-Rad, Hercules, CA), and probed with various primary antibodies (as described above). After incubation with alkaline phosphatase-conjugated secondary antibodies, specific proteins (apoptosis, NF-κB and MAPKs) were detected by chemiluminescence (Applied Biosystems, Foster City, CA).

Electrophoretic mobility shift assay

To assess NF-κB activation, nuclear extracts were prepared, and EMSAs were performed as described previously (Chaturvedi *et al*., 2000), with the following exceptions. In brief, nuclear extracts prepared from TNF-treated cells (2×10^6 cells ml^{-1}) were incubated with a ^{32}P-end-labelled 45-mer double-stranded oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long-terminal repeat 5′-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3′ (boldface indicates NF-κB-binding sites). After incubation for 30 min at 37°C, the DNA–protein complex was separated from free oligonucleotides in 6.6% native polyacrylamide gels. Specificity of binding of NF-κB to the DNA was also examined by competition with unlabelled oligonucleotide and binding with a double-stranded mutant oligonucleotide, 5′-TTGTTACAA **CTCACTTTC** CGCTG **CTCACTTTC** CAGGGAGGCGTGG-3′. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against the p50 or the p65 subunit of NF-κB for 30 min at 37°C before the complex was analysed by EMSA. The dried gels were visualized with a Phosphorimager scanner Storm 820 (Molecular Dynamics, Sunyvale, CA) and radioactive bands were quantified using ImageQuant software (GE Healthcare, Buckinghamshire, UK).

Nuclear factor-κB-dependent reporter gene expression assay

The effect of Lr-S 6475 on NF-κB-dependent reporter gene transcription induced by TNF was analysed by SEAP activity as described, with the following exceptions. To examine reporter gene expression, A293 cells (5×10^5 cells per well) were transiently transfected by the calcium phosphate method with the pNF-κB-SEAP plasmid (Invitrogen, Carlsbad, CA) and control pCMV-FLAG1 DNA plasmid for 24 h. Transfected cells were treated with Lr-S 6475 (20% v/v) for 16 h and stimulated with TNF (1 nM). The cell culture medium was harvested after 24 h of TNF treatment. The culture medium was analysed for SEAP activity according to the protocol described by the manufacturer (Clontech, Mountain View, CA) using a Victor 3 microplate reader at 570 nm (Perkin Elmer, Boston, MA).

Nuclear localization of p65 NF-κB by immunocytochemistry

Immunocytochemistry was used to examine the effect of Lr-S 6475 on the nuclear translocation of p65 as described previously (Shishodia and Aggarwal, 2004). Briefly, treated KBM-5 cells were plated on poly L-lysine-coated glass slides by centrifugation (Thermo Shandon; Cytospin 4), air-dried, and fixed with 4% paraformaldehyde after permeabilization with 0.2% Triton X-100. After being washed in PBS, the slides were blocked with 5% normal goat serum for 1 h and incubated with rabbit polyclonal anti-human p65 antibody at a 1/200 dilution. After overnight incubation at 4°C, the slides were washed, incubated with goat anti-rabbit IgG-Alexa Fluor 594 (Molecular Probes) at a 1/200 dilution for 1 h, and counterstained for nuclei with Hoechst 33342 (50 ng ml^{-1}) for 5 min. Stained slides were analysed by fluorescence microscopy (Labophot-2; Nikon). Pictures were captured using a Photometrics Coolsnap CF colour camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

Immunoprecipitation

KBM-5 cells (2×10^6 cells ml^{-1}) were pre-incubated with Lr-S 6475 (20% v/v) for 16 h, incubated with ALLN (50 μg ml^{-1}) for 30 min, and treated with TNF (1 nM) for 15 min. Cells were lysed for 30 min on ice in whole-cell extraction buffer [20 mM Hepes (pH 7.9), 50 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 0.5 mM EGTA, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 0.5 mM PMSF and 2 mM sodium orthovanadate]. Lysates containing 500 μg of proteins in extraction buffer were incubated with 1 μg ml^{-1} antibodies for 16 h. Immune complexes were precipitated using protein A/G-Sepharose beads for 1 h at 4°C. Beads were washed with extraction buffer and resuspended in SDS sample buffer, boiled for 5 min, and size-fractionated in SDS-PAGE.

Acknowledgements

The authors acknowledge Angela Major for her technical efforts with cytology and Tiffany Morgan for assistance with manuscript preparation. The authors acknowledge Eamonn Connolly (Biogaia AB) for provision of *L. reuteri* strains. This work was

supported by grants from the Crohn's and Colitis Foundation of America, Moran Foundation and the National Institutes of Health (RO1 DK065075, R21 AT003482). The authors also acknowledge support from the Texas Medical Center Digestive Diseases Center.

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