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Lactobacillus rhamnosus protects human colonic muscle from pathogen lipopolysaccharide-induced damage

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Key Messages

- *Lactobacillus rhamnosus* (LGG) interferes with inflammatory reaction triggered by infection in human colonic muscle. Therefore, this study provides a rational design for clinical studies in which the efficacy of probiotics in bacterial-related gut motor disorders could ultimately be determined.
- Aim of the study was to investigate the possible protective effects of LGG, and of its derived products (culture supernatant), on LPS-induced morphofunctional alterations of human colonic smooth muscle.
- Surgical specimen of human colon were used to obtained muscle strips and isolated cells. Experimental samples were exposed either to the sole LGG or to LPS, in the presence or absence of the probiotic or its supernatant.
- LGG, as well as its culture supernatant, activate an intrinsic myogenic response able to counteract LPS pro-inflammatory burst and to protect human gastrointestinal smooth muscle from LPS-induced damage. These effects occur via a direct involvement of TLR2 expressed on human colonic smooth muscle cells.

Abstract

Background *Lactobacillus species might positively affect gastrointestinal motility. These Gram-positive bacteria bind Toll-like receptor 2 (TLR2) that elicits anti-inflammatory activity and exerts protective effects on damage induced by lipopolysaccharide (LPS). Whether such effect occurs in gastrointestinal smooth muscle has not been established yet. Aim of this study was to characterize the effects of Lactobacillus rhamnosus GG (LGG) and of supernatants harvested from LGG cultures on human colonic smooth muscle and to explore their protective activity against LPS-induced myogenic morpho-functional alterations.* ***Methods*** *The effects of LGG (ATCC 53103 strain) and of supernatants have been tested on both human colonic smooth muscle strips and isolated cells in the absence or presence of LPS obtained from a pathogenic strain of Escherichia coli. Their effects on myogenic morpho-functional properties, on LPS-induced NFκB activation, and on cytokine production have been evaluated. Toll-like receptor 2 expression has been analyzed by qPCR and flow cytometry.* ***Key Results*** *Lactobacillus rhamnosus GG exerted negligible transient effects per se whereas it was capable of activating an intrinsic myogenic response counteracting LPS-induced alterations. In particular, both LGG and supernatants significantly reduced the LPS-induced morpho-functional alterations of muscle cells, i.e. cell shortening and inhibition of contractile response. They also hindered LPS-induced pro-inflammatory effects by decreasing*

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pro-inflammatory transcription factor NFκB activation and pro-inflammatory cytokine IL-6 secretion, and restored the secretion levels of anti-inflammatory cytokine IL10. ***Conclusions & Inferences*** *Taken together these data demonstrate that LGG protects human colonic smooth muscle from LPS-induced myogenic damage and might be beneficial on intestinal motor disorders due to bacterial infection.*

Keywords Lactobacillus rhamnosus *GG, human colonic smooth muscle, antipathogenic compounds, reversion of LPS damage, myogenic dysfunction, toll-like receptors.*

INTRODUCTION

Recent evidence suggests that neuromotor apparatus might represent a potential target for probiotics.[1] The *Escherichia coli* strain Nissle 1917 specifically modulates contractility of human colonic muscle strips,[2] and *Lactobacillus* species regulate jejunal motility,[3,4] human smooth muscle cell contractility,[5] and colonic neuron excitability.[6] Besides, the influence of microbiota on gastrointestinal motor activity has also been confirmed by observations in germ-free animals and models of gut dysfunction.[7–9] *Lactobacillus* species are Gram-positive bacteria whose recognition occurs through Toll-like receptor 2 (TLR2), which heterodimerizes with TLR1 and TLR6 to activate intracellular signaling.[10] Interestingly, these receptors have been found expressed by human colonic smooth muscle cells (SMC).[11] Toll-like receptor 2 function has also been reported in cardiomyocytes,[12] and its ligation has been shown to attenuate cardiac dysfunction in septic mice.[13] Furthermore, probiotics produce a vast array of biologically active substances, including many neuromodulators and neurotransmitters that influence afferent signaling to the enteric nervous system and to the brain[14–16] but also act on smooth muscle physiology.

Current evidence indicates that *Lactobacillus rhamnosus* (LGG) can exert anti-inflammatory effects in the gut and that some *Lactobacillus* species can specifically exert a protective activity against lipopolysaccharide (LPS)-induced inflammatory damage in murine models.[17–21] These effects have been reported on both small intestinal epithelial cells and ileum in rats,[22] and in an experimental model of septic shock in mice.[23] It is of note that, recently, it has been reported that also probiotic-derived factors released from living probiotics can demonstrate beneficial properties against pathogen-induced inflammation.[24] Thus, it has been suggested that probiotic supernatants could represent a valuable source of new antipathogenic compounds.[24]

In human colonic SMC, Gram-negative bacteria-derived LPS activates the pro-inflammatory TLR4 signaling pathway.[25] Prolonged activation of this pathway produces a persistent inflammatory cytopathic oxidative imbalance and consequent NF-κB activation that leads to the de-differentiation of SMC toward the so-called synthetic phenotype. This phenotype persists after LPS-washout and contributes to muscle dysfunction.[26] As *Lactobacillus paracasei* has been shown to normalize smooth muscle hyper-contractility in a murine postinfective irritable bowel syndrome model,[27] it can be hypothesized that these protective effects of *Lactobacillus* could occur, as in cardiomyocytes,[13] also in enteric muscle.

Aim of the present study was to study the direct effects of *L. rhamnosus* GG (LGG) and of supernatants harvested from LGG cultures on human colonic muscle to assess if they could exert a protective role against LPS-induced myogenic morpho-functional alterations.

MATERIAL AND METHODS

The following materials were used: Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin solution, gentamicin, anphotericin B, antimycin, fetal bovine serum (FBS) (LONZA, Basel, Switzerland); collagenase CLS type II (Invitrogen, Carlsbad, CA, USA); highly purified LPS obtained from a pathogen strain of *E. coli* (O111:B4) tested for the specific activation of TLR4 (Alexis, Lausen, Switzerland); ATP-regenerating system (Sigma Chemical Co., St Louis, MO, USA) and LGG obtained from the strain ATCC53103.

Preparation of human smooth muscle strips and cells and experimental protocols

Muscle specimens were obtained from disease-free surgical specimens of human colon. All patients gave informed consent, and the study was approved by Ethical Committee (ref. no. 1106, 2010). For muscle strips preparation, specimens were put in oxygenated, chilled Krebs solution containing (in mM) 116.6 NaCl, 21.9 $NaHCO_3$, 1.2 KH_2PO_4, 5.4 dextrose, 1.2 $MgCl_2$, 3.4 KCl, and 2.5 $CaCl_2$. After removal of the mucosa and submucosa layers, colonic circular smooth muscle was cut into small strips (10-mm long by 2-mm wide) by sharp dissection. The strips were mounted in separate 10-mL muscle chambers as previously described.[28] Strips were initially stretched to 2.0 g of force to bring them near conditions of optimum force development and equilibrated for an additional 30 min after continuous perfusion with oxygenated Krebs' solution. The solution was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 at a pH level of 7.4 and at 37 °C. During the perfusion period, spontaneous phasic contractions developed gradually and stabilized after a 30-minute period of equilibration. Isometric contractions were measured using force displacement transducers connected with a computer using MacLab system (Oxford, UK).

Primary human colonic SMC were isolated as previously described.[29] Briefly, slices of circular muscle layer were incubated overnight in DMEM supplemented with penicillin–streptomycin solution (10 000 U/mL), gentamicin (1 mg/mL), amphotericin B

(250 μg/mL), FBS (10%), an ATP-regenerating system (ATP 3 mM, phosphocreatine 10 mM, creatine phosphokinase 10 U/mL), antimycin (10 μM), and collagenase (150 U/mL). On the following day, digested muscle strips were suspended in DMEM supplemented only with FBS and antibiotics for 20 min to allow spontaneous dissociation of SMC. Cells were then harvested and used either immediately or maintained in suspension for up to 72 h.

Experimental protocols

Strips were exposed, for 30 min, only to Krebs solution (control) or to LGG at different concentrations and afterwards stimulated with a maximally effective dose of acetylcholine (10 μM).

The obtained richly pure and homogeneous primary SMC culture was exposed only to DMEM (control) or to different concentrations of LGG for 30 min or for 24 h. Culture was also exposed to 1 μg/mL highly purified LPS obtained from a pathogen strain of *E. coli* (O111:B4) in the presence or absence of the probiotic or its supernatant. Supernatants were harvested, by centrifugation and filter sterilization, from LGG culture.[30] At the end of the treatments, cells were prepared for the following analyses.

Total RNA extraction

Total RNA was harvested from SMC using Trizol (Invitrogen Life Technologies) according to the manufacturer's protocol, and RNA integrity was confirmed by electrophoresis on 1% agarose gel and ethidium bromide staining (0.1 μg/mL). Optical density at 260 nm was used to estimate the concentration of total RNA. RNA samples were stored at −70 °C.

Real-time analysis

After treatment at 37 °C for 30 min with 20–50 units of RNase-free DNase I (Roche Diagnostics Corp., Indianapolis, IN, USA), oligo-dT primers (Roche Diagnostics Corp.) were used to synthesize single-stranded cDNA. mRNAs were quantified using SYBR green Master Mix (Applera Corp., Norwalk, CT, USA) with specific human oligonucleotides in a GeneAmp Abiprism 7000 (Applera Corp.). In each assay, calibrated and no-template controls were included. Each sample was run in duplicate. SYBR green dye intensity was analyzed using the Abiprism 7000 SDS software (Applera Corp.). All results were normalized to the unaffected housekeeping gene actin. The following specific human oligonucleotides were used: TLR1: 5′-GCC TAT ATG CAA AGA GTT TGG C-3′, 5′-CTC TCC TAA GAC CAG CAA GAC C-3′. **TLR2**: 5′-GCC AAA GCT TTG ATT GAT TGG-3′, 5′-TTG AAG TTC AGC TCC TG-3′. **TLR6**: 5′-CCC ATT CCA CAG AAC AGC AT-3′, 5′-ATA AGT CCG CTG CGT CAT GA-3′. ***β*-ACTIN**: 5′-TCA CCC ACA CTG TGC CCA TCT ACG-3′, 5′-CAG CGG AAC C. C TCATTG CCC AAT G-3′

Measurement of contractile response

Contraction was measured on SMC by image scanning micrometry using a ProgRes® camera with CapturePro 2.6 application software (Jenoptik Laser Optik, Jena, Germany) installed on a contrast-phase microscope Leica 2500 (Leica Microsystems, Wetzlar, Germany) as previously described.[31] Biological morpho-functional features (cell length and contractile response) were measured in blind both in the untreated state (control) and upon LGG or LPS exposure. Contraction to a maximal dose of acetylcholine (1 μM) was expressed as percentage decrease in cell length from control taken as 100.

Surface expression of TLR2

An indirect immunofluorescence assay was developed on SMC to quantify surface expression level of TLR2.[32] Unfixed living cells were incubated for 30 min at 4 °C with monoclonal anti-TLR2 (0.1 μg/mL) in PBS containing 1% BSA. After washings in PBS, fluorescein isothiocyanate-conjugated anti-mouse IgG (γ-chain specific, Sigma-Aldrich, St Louis, MO, USA) was then added and incubated at 4 °C for 30 min. After further washings, cells were counterstained with Trypan blue dye to distinguish dead cells (excluded from our analysis) from living cells. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose′, CA, USA) using FL-1 and FL-3 detectors.

NFκB assay

Quantitative analysis of the activation state of nuclear factor κB (NFκB) was performed using specific ELISA kit, FACE NFκB p65 profiler (Active Motif, Rixensart, Belgium) following manufacturer instruction.

Analysis of cytokine secretion

IL-6 and IL-10 levels were determined in supernatants of control and treated cells using sensitive ELISA kits (R&D System, Minneapolis, MN, USA). Briefly, supernatant aliquots were removed at the end of treatments and assayed for IL-6 and IL-10 presence according to the manufacturer's instructions.

Data and statistical analysis

Data are expressed as mean ± SE of duplicate examinations of n experiments, n referring to the number of individual patients from whom the colonic specimens were obtained. Statistical analysis was performed by parametric ANOVA test, corrected for multiple comparison by the Bonferroni procedure. p values of less than 0.05 were considered as significant.

RESULTS

To assess the interaction of LGG with human colonic smooth muscle, whole muscle tissue (muscle strips, MS) and primary cultures of isolated SMC were exposed to increasing concentrations of the probiotic. At rest, in the absence of LGG, MS developed a stable phasic contraction with standard contractile response to a maximal dose of acetylcholine of 42.5% ± 7.6 over basal, while SMC presented a resting cell length of 90.7 ± 1.8 μm and a maximal contractile response to acetylcholine of 31.1% ± 1.1 over basal. Short-term exposure (30 min) of both MS and SMC to LGG determined significant ($p < 0.05$) dual effects (Fig. 1A and B). Firstly it induced a dose-dependent increase, in

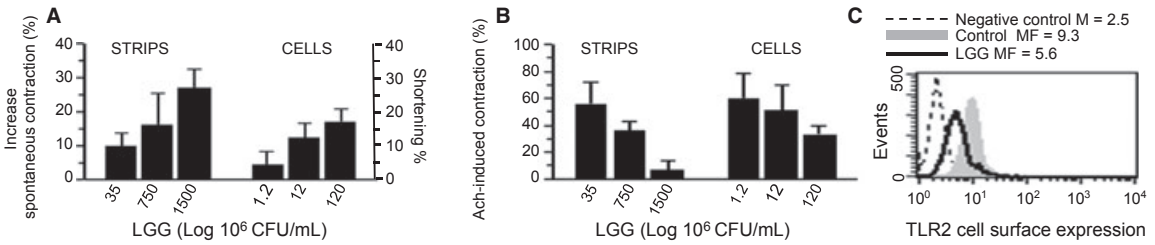

Figure 1 Short-term effects (30 min) of LGG on human colonic smooth muscle. (A) LGG dose-dependent increase in muscle strips 'spontaneous' contraction (Left *y* axis) and smooth muscle cells (SMC) shortening (Right *y* axis). Data are the mean ± SE of five independent experiments. (B) LGG dose-dependent decrease in contraction induced by muscarinic agonist acetylcholine (Ach) 1 μM of muscle strips (Left columns) and SMC (Right columns). Data are the mean ± SE of five independent experiments. (C) Flow cytometry analysis of TLR2 expression on SMC before and after exposure to 120 × 10^6 CFU/mL of LGG. Results obtained in a representative experiment are shown. Numbers represent the median fluorescence intensity (M). Empty dashed histogram: negative control; full light gray histogram: untreated control cells; empty black histogram: cells incubated with LGG.

respect to resting state, of spontaneous contraction in MS (Fig. 1A, left *y* axis) and a length decrease (shortening) in SMC (Fig. 1A, right *y* axis). The second motor modification induced by LGG consisted in alterations of smooth muscle activity ending in the inhibition of contractile response to the muscarinic agonist acetylcholine. Again, LGG effects were observed both on MS and isolated SMC and increased dose-dependently, strongly suggesting a direct myogenic effect of LGG (Fig. 1B). Individual results of dose- and time-dependent effects of LGG on SMC are shown in Figs S1 and S2, respectively. Representative tracing of human muscle strips are shown in Fig. S3.

To verify this hypothesis, the quantitative expression of Gram-positive bacteria-sensing receptors, namely TLR2 and its two heterodimers TLR1 and TLR6, was evaluated. qPCR analysis showed that the mRNA encoding TLR1, 2, and 6 receptors were constitutively expressed on SMC in almost similar amounts (TLR1: 7.46 ± 0.90; TLR2: 7.19 ± 0.37; TLR6: 7.41 ± 0.33). Thereafter, a quantitative analysis of cell surface expression level of TLR2 was also performed, before and after SMC exposure to 120 × 10^6 CFU/mL of LGG. Surface expression of TLR2 in resting cells (median fluorescence intensity, MFI: 8.96 ± 0.44) was significantly decreased (−51.1 ± 11.3%) in cells exposed to LGG (MFI: 4.42 ± 1.65, $p < 0.01$; Fig. 1C). This reduction in the receptors available for monoclonal anti-TLR2 binding further suggested the occurrence of an interaction of LGG with TLR2 receptors.

Lactobacillus rhamnosus GG-induced morpho-functional smooth muscle modifications were reversible. In fact, SMC shortening (Fig. 2A) and reduced contractility (Fig. 2B), observed at shorter time points (30 and 60 min), were reverted at prolonged exposure times (24 h) in that SMC, despite the presence of LGG, progressively restored their length and their contractile response to acetylcholine. Further evidence of the reversibility of short-term LGG effects was offered by the complete recovery of stable phasic contraction and contractile response to acetylcholine in MS following LGG washout (Fig. 2C).

To test LGG protective role against pathogenic infective bursts, SMC were incubated with pathogen LPS (*E. coli* O111:B4) in the absence or presence of LGG. As LGG by itself modified morpho-functional

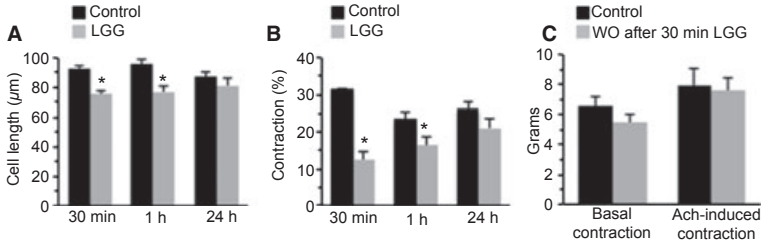

Figure 2 Time-dependency of LGG effects on colonic human smooth muscle. (A and B) LGG effects on smooth muscle cells length (A) and acetylcholine-induced contractile response (B). Cells were incubated for increasing time with LGG (120 × 10^6 CFU/mL) (Controls, black columns; LGG, gray columns). Data are the mean ± SE of six independent experiments. *$p < 0.05$ *vs* untreated control cells. (C) Spontaneous (Basal) and acetylcholine (Ach)-induced contraction (1 μM) of muscle strips before (Control, black columns) and after LGG washout (WO, gray columns). Data are the mean ± SE of five independent experiments.

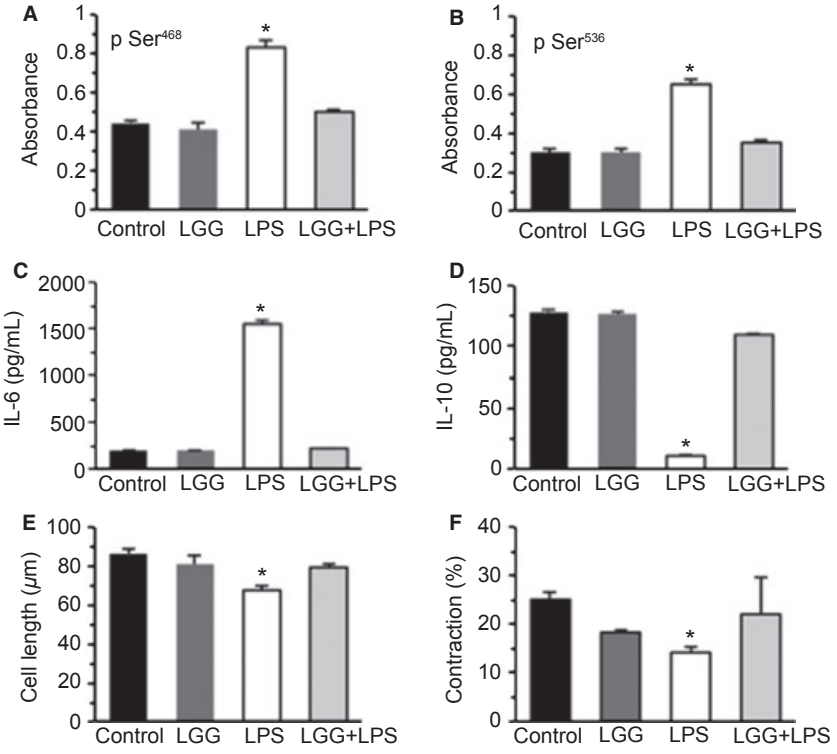

Figure 3 Long-term effects of LGG on human smooth muscle cells (SMC). Smooth muscle cells were incubated for 24 h with LGG (120×10^6 CFU/mL, deep gray columns) or with 1 μg/mL LPS in the absence (white columns) or presence of LGG (light gray columns). Control samples: black columns. (A and B) Phosphorylation of NFκB subunits Ser^{468} (A) and Ser^{536} (B). Data are the mean ± SE of four independent experiments. $^{*}p < 0.01$ *vs* LGG+LPS. (C and D) Quantitative ELISA of IL-6 (C) and IL-10 (D) release. Data are the mean ± SE of three independent experiments. $^{*}p < 0.05$ *vs* LGG+LPS. (E and F) Evaluation of SMC length and contraction. Data are the mean ± SE of three independent experiments. $^{*}p < 0.01$ *vs* LGG+LPS.

parameters up to 1 h-exposure, the effect of LGG on LPS-induced SMC alterations was evaluated after 24 h incubation. *Lactobacillus rhamnosus* GG, which *per se* did not modify the phosphorylation of p65 NFκB subunits Ser^{468} and Ser^{536}, was able to hinder LPS-induced NFκB activation up to 83.8% ± 3.9 for Ser^{468} and 85.7% ± 4.3 for Ser^{536} (Fig. 3A and B). Furthermore, LPS-induced increased secretion of IL6 (668.7% ± 12.3) and parallel decreased secretion of IL10 (−42.1% ± 2.3) were counteracted by LGG (Fig. 3C and D). Finally, as far as morpho-functional alterations were concerned, cell shortening and inhibition of acetylcholine-induced maximal contraction observed after a 24 h-exposure of SMC to LPS were significantly reduced in the presence of LGG (26.1 ± 2.6% and 44.9 ± 5.5%, respectively, Fig. 3E and F). In fact, LPS-induced cell shortening was reversed by 59.9 ± 6.2% whereas inhibition of contraction by 89.0 ± 10.9%. These long-lasting LGG effects were coupled to a decrease in surface expression of TLR2 (MFI: resting cells 9.3 ± 2.3; 24 h-LGG exposed cells 6.2 ± 1.9) suggesting the occurrence of a persistent interaction of LGG with TLR2 receptors.

To evaluate the possible anti-inflammatory effects of LGG-derived factors released during culture, SMC were exposed for 24 h to LPS in the presence or absence of cell-free supernatants harvested from LGG culture (S-LGG). Similar to LGG, supernatants *per se* did not modify the phosphorylation of p65 NFκB subunits Ser^{468} and Ser^{536} but were able to counteract LPS and to restore NFκB subunits phosphorylation at control levels (Fig. 4A). In parallel, supernatants were able to neutralize LPS-induced alterations of IL6 and IL10 secretions (Fig. 4B) and to restore cell length and contraction (Fig. 4C). The effects of LGG supernatants occurred through the interaction with TLR2 as, similar to LGG, surface expression of TLR2 was significantly decreased in cells exposed to supernatants (MFI: resting cells 9.4 ± 1.3; S-LGG: 6.9 ± 0.6) (Fig. 4D).

DISCUSSION

This study suggests that both LGG and its supernatants directly affect human colonic smooth muscle through the direct activation of the Gram-positive sensing TLRs. The outcome was different depending on exposure times. Initially, LGG induced transitory myogenic changes with alterations in morpho-functional parameters, either in the whole muscle tissue or in isolated cells. Afterwards, it activated an intrinsic myogenic response that was apt to counteract pro-inflammatory burst protecting human gastrointestinal smooth muscle from pathogen LPS-induced damage. These latter effects were also mimicked by LGG

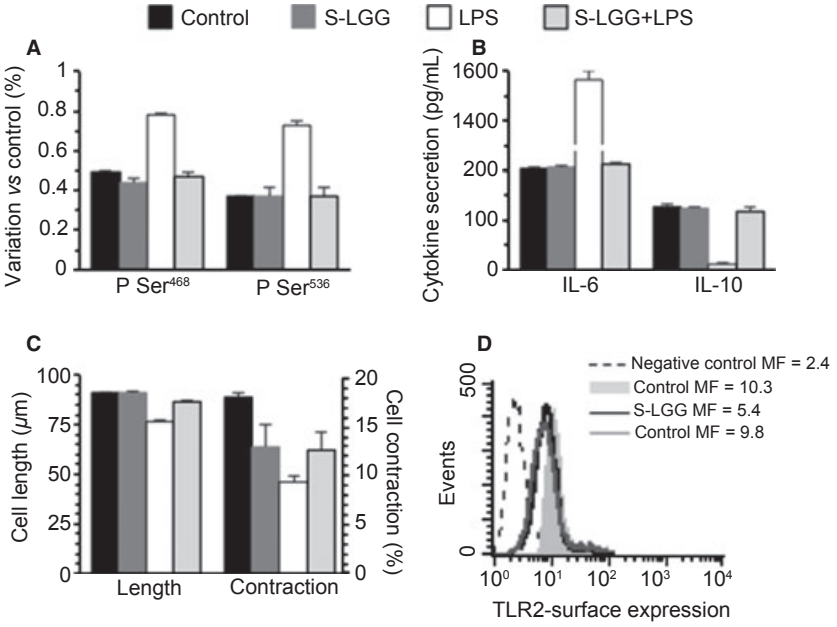

Figure 4 Effects of supernatants harvested from LGG cultures on human smooth muscle cells. Smooth muscle cells were incubated for 24 h with supernatants harvested from LGG cultures (S-LGG, deep gray columns) or with 1 μg/mLLPS in the absence (white columns) or presence of supernatants (light gray columns). Control samples: black columns. (A) Phosphorylation of NFκB subunits Ser^{468} and Ser^{536}. Data are the mean ± SE of three independent experiments. $^{\star}p < 0.01$ *vs* S-LGG+LPS. (B) Quantitative ELISA of IL-6 and IL-10 release. Data are the mean ± SE of three independent experiments. $^{\star}p < 0.05$ *vs* S-LGG+LPS. (C) Evaluation of SMC length and acetylcholine-induced contraction. Data are the mean ± SE of three independent experiments. $^{\star}p < 0.05$ *vs* S-LGG+LPS. (D) Flow cytometry analysis of TLR2 expression on SMC before and after exposure to S-LGG. Results obtained in a representative experiment are shown. Numbers represent the median fluorescence intensity (MF). Empty dashed histogram: negative control; full light gray histogram: untreated control cells; empty black histogram: cells incubated with S-LGG.

culture supernatants, suggesting that probiotic-derived factors might exert an anti-inflammatory activity, opening new therapeutic strategies for bacterial-related intestinal motor disorders.

The direct effect of LGG on human colonic muscle was supported by several lines of evidence. Firstly, similar expression levels either of TLR2 or of its two heterodimers, namely TLR1 and TLR6, were observed in human colonic SMC. On the other hand, cell surface expression of TLR2 decreased when human SMC were exposed to LGG. The absence of available receptors for monoclonal anti-TLR2 binding indirectly substantiated the interaction of LGG with membrane receptors. The observed decrease of TLR2 expression on SMC following LGG might indicate that the binding of LGG to TLR2 could 'conceal' the immunogenic epitope recognized by the specific antibody but, also, a down-modulation of TLR2 surface expression. This could be due to the internalization of receptor/ligand association as a consequence of receptor recycling mechanism. Secondly, evidence for a direct action of LGG on colonic muscle came from the dose-dependency of LGG effects detected on both isolated SMC and intact tissue. This led to an increase in spontaneous contraction in muscle strips, likely related to the cell-length shortening observed at cellular level, and to the inhibition of the contractile response induced by the muscarinic agonist acetylcholine. The increased contraction on ileal strips in guinea-pig[3] and the accelerated ileal transit in germ-free rats[9] induced by different species of *Lactobacillus* previously reported can then be ascribed to probiotics effects on enteric muscle. Besides, the use of primary human SMC culture has allowed to demonstrate that LGG could influence muscular activity in a reversible manner. Indeed LGG-induced morpho-functional smooth muscle modifications lasted less than 24 h, despite the continuous presence of LGG, out of keeping with what previously observed with LPS on human colonic SMC.[26] These dual, time-dependent effects have previously been observed exposing human SMC to TLR2 synthetic ligands (namely synthetic diacetylated and triacetylated lipopeptides, PAM2- and PAM3-CSKA) that bind, respectively, the heterodimers TLR2/TLR6 and TLR2/TLR1[11] indicating their dependency on TLR2 activation. The prolonged activation of TLR2 by LGG, as indicated by the persistent decrease in their cell surface expression at 24 h, likely activates

the anti-inflammatory PI3K/AKT signaling pathway that is known to be responsible of the beneficial effects of TLR2 ligands through the time-dependent regulation of the NFκB pathway on both mice cardiac cells[13] and rabbit colonic smooth muscle.[33] Indeed TLR2, beyond the MyD88 binding motif, shared with all the others TLRs receptors, contains a PI3K binding motif, also present in TLR1 and TLR6 but missing in other TLRs,[34] that activates this cascade.[35]

Onward, LGG protected human SMC from LPS-induced damage and this likely occurred through LGG binding to TLR2 whose activation leads to IL10-mediated anti-inflammatory effects. IL10 is a major immunosuppressive and anti-inflammatory factor, essential for the homeostatic control of infection and inflammation.[36] Its release by peripheral blood mononuclear cell levels is reduced in irritable bowel syndrome patients and reverted to levels observed in healthy volunteers after probiotic administration.[37] Preventive effects on LPS-induced damage have already been reported either for different *Lactobacillus* species[19,22] or for TLR2 activation.[13,38] Toll-like receptor 2 is a potent IL-10 inducer, through which it abolishes chronic inflammation by modulating cell signaling associated with TLRs in epithelial and dendritic cells.[39,40] In human SMC, protection exerted by LGG against LPS-induced damage occurred through the inhibition of both NFκB subunits phosphorylation and pro-inflammatory cytokine IL6 secretion upon activation of surface TLR2 whose expression was reduced after LGG exposure. Similar down-regulation of pro-inflammatory cytokine release by Lactobacilli has been previously reported in human intestinal dendritic cells challenged with *Salmonella*[41] and in human peripheral blood monocytes-derived macrophages primed by LPS.[42] It is then likely that also in human colonic SMC, TLR2 receptor, upon its activation by LGG and derived products, might counteract LPS-induced morpho-functional myogenic alterations. In agreement with the present data are the observation that *L. paracasei* normalized muscle hypercontractility in a murine model of postinfective gut dysfunction.[1] Evaluation of protective effect of probiotics might be a powerful tool for the screening probiotic strains of possible use in human health.[43] Furthermore, it would be also worthwhile to assess if, similar to antioxidants,[26] LGG protective effects persist even if administered after LPS exposure.

Finally, it has to be noted that LGG myogenic antipathogenic effects might be mediated by LGG-derived factors as protective effects against LPS-induced damage were also observed in SMC exposed to the sole LGG culture supernatants. Again, these effects occurred through interaction with membrane TLR2 receptors. Similar TLR-mediated anti-inflammatory effects have been reported for cell-free culture supernatants of *L. paracasei*[41] and *Bifidobacterium breve*[44] on human dendritic cells challenged with *Salmonella typhi*. The beneficial effects of probiotic-derived factors have been less documented than that of probiotics. Only in the recent years some studies, dealing with their beneficial properties against pathogen-induced inflammation and related alteration of cytokine release, have been carried out.[24] The identification of probiotic biofactors with beneficial effects could disclose new therapeutic strategies for a wide range of digestive diseases, avoiding risks related to the administration of live bacteria. In the meanwhile, the potential therapeutic value of probiotics in functional gut disorders and the evidence that motor and neural apparatus represent a potential target for probiotics in gut postinfective disorders has already been suggested by studies carried out in animal models.[27]

In conclusion, this study provides novel insights about the possibility that LGG could reduce the risk of progression to a postinfective disorder by interfering with the inflammatory reaction triggered by infection. In fact, LGG and its secreted products seem to be able to directly protect human colonic smooth muscle from LPS-induced myogenic damage through the interaction with TLR2. These results can provide a rational design for clinical studies in which the efficacy of probiotics in bacterial-related gut motor disorders could ultimately be determined.

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DISCLOSURE

No competing interests declared.

AUTHOR CONTRIBUTION

FA conducted the study, participated in the design of the study and analysis of data; AS performed cell isolation, primary cultures and qPCR; AA performed muscle strips experiments; PM conducted the immunofluorescence analysis; CP performed cell treatments and participated in analysis of data; RC and PC provided surgical specimens; MG and CC participated in the design of the study and revision of the manuscript; MM provided study supervision and critical revision of the manuscript for important intellectual content; WM and CS designed and coordinated the study and drafted the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Individual evolution of short-term (30 min) dose-dependent LGG effects on cell length (A) and acetylcholine (Ach)-induced contraction (B) of human colonic smooth muscle cells.

Figure S2. Individual evolution of time-dependent LGG (120×10^6 CFU/mL) effects on cell length (A) and acetylcholine (Ach)-induced contraction (B) of human colonic smooth muscle cells.

Figure S3. Representative tracings of human smooth muscle strips before and after LGG exposure.