The Role of Probiotics in Lipopolysaccharide-Induced Autophagy in Intestinal Epithelial Cells

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Key Words
Autophagy • Lipopolysaccharide (LPS) • Probiotics • Inflammatory bowel disease (IBD)

Abstract

Background/Aims: Dysfunction of autophagy has been associated with loss of intestinal homeostasis. Lipopolysaccharide (LPS) from Gram-negative bacteria is known to be a major initiator of intestinal epithelial cell (IEC) autophagy. Although probiotics have been recognized to be involved in many therapeutic properties and participate in host defense responses, the molecular mechanisms by which probiotics exert these positive effects remain unknown. This study assessed the effect of probiotics on LPS-induced physical barrier dysfunction and the underlying mechanism of probiotic action in IECs with a focus on autophagy. Methods: A LPS-induced autophagic model was established in rat IEC18 cells wherein cells were treated with culture medium supernatants of Bifidobacteria following LPS intervention at indicated times. Autophagosomes in IEC18 cells were visualized by confocal microscopy after transfection with a tandem GFP-mCherry-LC3 construct and also by transmission electron microscopy. Autophagy-associated protein levels were analyzed by western blot and transepithelial electrical resistance (TEER) was measured using an epithelial voltohmmeter. Results: Probiotic treatment could effectively inhibit LPS-induced autophagy, as evidenced by the decreased ratio of microtubule-associated light chain 3 (LC3)-II/LC3-I, fewer autophagic vacuoles, and reduced punctate distribution of GFP-mCherry-LC3. In addition, probiotics prevented chloroquine (CQ) inhibition of autophagic flux and autophagolysosomal fusion as indicated by a failure to recruit LAMP1 and cathepsin D to lysosomes. Interestingly, ATG16L1 knockdown did not inhibit the effect of probiotics on LPS-induced autophagy. Furthermore, the diminished barrier function could be prevented by probiotics. Conclusions: We provide evidence that autophagy mediation by probiotics may be involved in enteroprotection against LPS-induced intestinal epithelial toxicity, and could serve as a novel mechanism through which probiotics promote and maintain gut homeostasis.

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**Introduction**

Autophagy is the primary intracellular catabolic process wherein malfunctioning cytoplasmic proteins or disabled organelles are delivered to the lysosomes for degradation and recycling [1, 2]. Basal levels of autophagy have been demonstrated to be involved in certain types of physiologic conditions. When cells are exposed to certain internal and external environment stimuli such as nutritional stress or invasion by microbial or viral pathogens, increased autophagy is induced to maintain cell survival [1, 3]. Autophagy also plays a crucial mediating role in the development of various pathologies in humans, including neurodegeneration [4], tumor formation [5, 6], and cardiovascular disease [7].

In contrast, excessive autophagy can contribute to pathological conditions and to autophagic cell death [8]. Suppression of excess autophagy could alleviate both acute cardiac injury and promote survival of recipient rats in a LPS-induced cardiomyocyte contractile dysfunction model [9]. Moreover, a study by Bi et al. [10] reported that induction of autophagy promoted death of rat renal glomerular mesangial cells, while in hepatocytes, pretreatment with wortmannin could alleviate lipopolysaccharide/D-galactosamine-induced acute hepatocytotoxicity and reduce apoptosis and necrosis through inhibition of autophagy [11]. These results indicate that induction of excessive autophagy may be harmful and could aggravate injury to organs.

Recent accumulating evidence showed a close association between autophagy in intestinal epithelial cells and inflammatory bowel diseases (IBDs). Indeed, single nucleotide polymorphisms (SNPs) within two autophagy-related genes, autophagy-related 16-like 1 (ATG16L1) and the immunity-related GTPase family M (IRGM), are important loci for IBD pathogenesis [12, 13]. Dysfunction of these autophagy-related proteins is associated with survival of intracellular bacteria, pro-inflammatory cytokine secretion, and colitis onset [12, 14]. Thus, controlling how autophagy is regulated might be a critical strategy for IBD prevention and therapy.

Probiotic bacteria are known to suppress pathogenic bacterial growth, as well as modulate both intestinal commensal microbiota [15, 16] and the mucosal immune response [17, 18]. Oral administration of specific probiotics can reduce experimental colitis in IL-10-deficient mice [19], and in clinical studies probiotics affected gut flora so as to prevent or treat different gut disorders ranging from diarrhea and pouchitis to chronic relapsing IBD [20, 21]. However, the molecular mechanisms by which probiotics exert positive effects on gut homeostasis remains unknown.

*Bifidobacterium bifidum* is a group of probiotic bacterial strains that has been verified to be effective in preventative and maintenance treatment of diarrhea and colitis [22-24]. Thus, in this study we used this clinically validated probiotic to identify at a molecular level relevant biological processes and functional changes that occur in response to therapeutic manipulation of gut inflammation in an in vitro model using cultured rat intestinal epithelial cells (IECs). The aim of this research was to investigate the effect of probiotics on LPS-induced autophagy and to provide novel insight into probiotic-mediated intestinal protection. Our results demonstrate that probiotics could effectively alleviate LPS-induced autophagy through inhibitory effects during the three stages of autophagy: initiation, maturation, and degradation. More importantly, we found that probiotics can significantly ameliorate and restore physical barrier integrity that is diminished by LPS treatment.

**Materials and Methods**

**Cell and bacterial strain culture**

IEC18 cells (passage 20-40) originating from rat ileum epithelium were obtained from the American Type Culture Collection (Manassas, VA, USA; ATCC catalog no. CRL-1589). Maintenance medium was Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and 110 mg/L sodium pyruvate, supplemented with 5% fetal bovine serum (Gibco® Cell Culture, Melbourne, VIC, Australia) and 0.1 U/ml bovine insulin (Sigma, St. Louis, MO, USA). Cells were cultured at 37°C in 5% CO₂ at 90%
relative humidity. Supernatants from culture medium of the probiotic Biﬁdobacterium bisﬁdum (B. bisﬁdum, ATCC29521) were a gift from the Wuhan Research Institute of First Light Industry (Wuhan, Hubei Province, China). Supernatants were extracted from 5 × 10⁸ colony-forming unit (CFU)/L) B. bisﬁdum cultures and centrifuged, ﬁltered, and stored at −20°C until analysis.

Cell culture stimulation and bacterial treatment
Conﬂuent IEC18 cell monolayers were stimulated with a mild dose of 5 μg/mL Escherichia coli (E. coli) LPS (O111:B4, Sigma, St. Louis, MO, USA) at the indicated times. To assess the role of probiotics on LPS-induced autophagy, B. bisﬁdum supernatants were used at a multiplicity of infection (MOI) of 0.1, meaning that the ratio is one CFU of bacteria to 10 IEC18 cells.

RNA extraction and real-time quantitative polymerase chain reaction
Cells were collected at the indicated time points. Total RNA was extracted from the collected cells with TRIZol reagent (Invitrogen) according to the manufacturer’s instructions and reverse transcribed to complementary DNA using Multiscribe reverse transcriptase (Applied Biosystems). Real-time quantitative PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen) and a StepOne Real-Time PCR apparatus (Applied Biosystems). Primer sequences used for autophagy-related genes are described in Table 1. The expression level of the autophagy-related genes was normalized relative to levels of the housekeeping gene GAPDH, which was used as an endogenous control.

Protein preparation and western blot analysis
Protein preparation and western blotting were performed as previously described [25]. Briefly, cells were harvested and lysed in ice-cold RIPA lysis buffer (Beyotime, Nantong, China) for 30 min before centrifugation at 12,000 rpm for 15 minutes. The protein concentration of the resulting supernatants was quantiﬁed using the BCA Protein Assay Kit (Beyotime). For western blot analysis, equal amounts (40μg) of protein were separated by 10% or 12% tris-glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred to a polyvinylideneﬂuoride (PVDF) membrane (Millipore, MA, USA). After the blockage step of unspeciﬁc binding sites, the membranes were probed with the primary antibody LC3 (1:2,000 dilution; Cell Signaling Technology, Danvers, MA, USA or 1:1,000 dilution; AB Clonal Technology, Boston, MA USA), anti-p62/SQSTM1 (1:2,000 dilution; MBL International Corporation), anti-lysosome-associated membrane protein 1 (LAMP1) (1:1,000 dilution; ab24170, Abcam) and anti-cathepsin D (1:500 dilution; sc-6486; Santa Cruz) at 4°C overnight. Following incubation with corresponding secondary antibodies (1:2,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h on a rotating platform, bands were developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientiﬁc) and intensities were quantiﬁed by densitometric analysis. The ratio of LC3-II/LC3-I was calculated to determine the activated expression of LC3.

Transmission electron microscopy (TEM)
After the indicated stimulation period, cells were ﬁxed with 2.5% phosphate-buffered glutaraldehyde, post-ﬁxed in 1% osmium tetroxide for 1 h, rinsed with 0.1 M phosphate buffer (pH 7.4), and then dehydrated with increasingly graded alcohols before embedding in Epon 812 (SPI Supplies, West Chester, PA, USA).

Table 1. Primers used for autophagy-related genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>ATG5</td>
<td>ATACTCTGTCTTCTGCTGTCTT</td>
<td>CAAGCTAGCTCACGACTCAATG</td>
</tr>
<tr>
<td>ATG7</td>
<td>TGGTGAGAGCGTGAAGACC</td>
<td>GATTCACAGAGACGGGAC</td>
</tr>
<tr>
<td>ATG12</td>
<td>GGCTACAGAGTAAAGCTGTCA</td>
<td>GTGAACATGTTGGTCCACAGCA</td>
</tr>
<tr>
<td>ATG16L1</td>
<td>ATCTTTTGGGAGAGGCTTGTTG</td>
<td>CTGCAAGCTGACTCTCGTCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCTCCAGGAGGAGATCCC</td>
<td>TTCAGGTAAGCCACAGGCTT</td>
</tr>
</tbody>
</table>
Sections or grids were observed using a FEI Tecnai G² 12 TEM (FEI Company, Hillsboro, USA). The number of autophagic vacuoles was calculated and analyzed by a pathologist who was blinded to the sample identity.

**GFP-mCherry-LC3 plasmid transfection and confocal microscopy**

Logarithmic growth phase IEC18 cells were seeded on coverslips at 60-80% confluence and transfected with GFP-mCherry-LC3 plasmid DNA (Changsha Yingrun Biotechnology Co., Ltd. Changsha, Hunan province, China) using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer’s instructions. After transfection for 6 h, the medium was replaced with fresh culture medium and the cells were cultured for an additional 24 hours before stimulation with LPS in the absence or presence of probiotics. The cells were then washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were harvested and mounted on coverslips for imaging with an A1Rsi/A1si spectral imaging confocal system (Nikon, Tochigi, Japan). Autophagy was evaluated by the formation of punctate fluorescent structures.

**RNA interference**

Short hairpin RNA (shRNA) against ATG16L1 was transfected into IEC18 cells with Lipofectamine 2000. After 24 hours of incubation, the effectiveness of expression inhibition was evaluated by western blot. The shRNA candidate target sequence for ATG16L1 was 5′-GGA GAT ACA GAC GAA TGA AGC-3′. A scrambled shRNA sequence, 5′-GTT CTC CGA ACG TGT CACGT-3′, was used as a negative control (NC-siRNA).

**Measurement of transepithelial electrical resistance (TEER)**

For TEER experiments, cells were plated into the apical chamber of the inserts (1.12 cm² area, 0.4 μm pore size; Corning, NY, USA) at a density of 1-2 x 10⁵ cells/insert, and cultured 2 weeks. The formation of polarized confluent monolayers and barrier properties were monitored by measuring the TEER with an EVOM TEER meter (World Precision Instruments, Sarasota, FL, USA). When monolayers achieved a steady state of > 40 Ω·cm² [26], which indicated development of functional polarity and an intact monolayer, TEER readings were acquired at 2 h intervals after infection.

**Results**

**Lipopolysaccharide infection, but not probiotic treatment, induces autophagy in IEC18 cells**

To evaluate the effect of LPS administration on autophagy in the intestinal epithelium, IEC18 rat epithelium cells were treated with a mild dose of LPS (5 μg/mL) at 2 h time intervals. Quantitative PCR (qPCR) analysis of mRNA levels of several autophagy-related genes, including ATG5, ATG7, ATG12, and ATG16L1 showed that the expression levels of these genes gradually increased as the LPS treatment time increased from 2 h to 6 h, to reach a peak level at 6 h, at which time the levels gradually decreased (from 6 h to 24 h; Fig. 1A-D). In contrast, for cells that were treated with *B. bifidum* culture supernatants alone, the basal expression level of these genes was slightly, although not significantly, higher than that in the corresponding control (Fig. 2A-D), indicating that probiotic treatment alone had no remarkable effect on the expression of genes involved in intestinal autophagy.

**Probiotic treatment inhibits increased autophagy induced by LPS**

During the autophagy process, damaged macromolecules and organelles are first sequestered in double-membrane vesicles called autophagosomes. The autophagosomes
then fuse with lysosomes to form autolysosomes, the contents of which are degraded by acidic lysosomal hydrolases [27]. Microtubule-associated light chain 3 (LC3) is initially present in the cytoplasm as LC3-I before modification to a phosphatidylethanolamine (PE)-conjugated membrane-bound form to produce LC3-II, which is finally recruited to the autophagosomes [28, 29]. Thus, transformation of LC3-I to LC3-II is essential for the formation of autophagosomes and is a widely accepted molecular marker for autophagy activation. To assess whether probiotics influenced LPS-induced autophagy, we chose autophagy rise period (0-6 h) for further experiments. That is, IEC18 cells were first exposed to LPS for 2 or 4 h before incubation with \textit{B. bifidum} supernatants harvested and assayed for LC3 concentrations. Western blotting revealed that after exposure to LPS from 0-6 h, a significant up-regulation of LC3-II protein expression and an increase in the ratio of LC3-II to LC3-I (LC3-II/LC3-I) were observed but occurred a rapid time-dependent decrease after probiotic treatment for 4 h. (Fig. 2E, F). Similarly, probiotics inhibited the increases in mRNA expression levels of \textit{ATG5, ATG7, ATG12}, and \textit{ATG16L1}, which were elevated by LPS treatment relative to the corresponding control groups (Fig. 2A-D).

**Fig. 1.** Time dependence of LPS-induced effects on autophagy in IEC18 cells. (A-D) Expression levels of autophagy-related genes \textit{ATG5, ATG7, ATG12}, and \textit{ATG16L1} were up-regulated in LPS-treated cells relative to control cells (at 0 h) that were treated with PBS only. Results are shown from five independent experiments. * p < 0.05 and ** p < 0.01 vs. control.

Probiotic treatment suppressed cytoplasmic vacuolization and mitochondrial damage during LPS administration

The effect of probiotics on LPS-induced autophagy was also verified by electron microscopy analysis at the ultrastructural level. As shown by TEM, there were fewer autophagic vacuoles in the unstimulated and probiotic-treated groups (Fig. 3A-B). In LPS-treated intestinal epithelia, autophagosome structures were more prevalent and mitochondria were severely swollen or significantly deformed (Fig. 3C). After the addition of probiotic supernatants, less cytoplasmic vacuolization was observed in the group exposed to the supernatants for 4 hours as compared to cells treated for 2 hours or cells treated with...
Fig. 2. Probiotics suppress LPS-induced autophagy activation in IEC18 cells. (A-D). Quantitative PCR showed that the expression levels of autophagy-related genes ATG5, ATG7, ATG12, and ATG16L1 were slightly up-regulated in *Bifidobacterium*-treated cells compared to those in the untreated group, although the differences were not statistically significant. The combination of incubation time as shown in the X axis. Expression levels were significantly increased in cells treated with LPS for 4 h or 6 h (namely, LPS 4 h or 6 h and B.bifidum 0 h), but were down-regulated completely by supernatants of *Bifidobacterium* cultures treated for 4h after LPS intervention for 2 h (namely, LPS 6 h and B.bifidum 4 h). (E) Western blot analysis of LC3 levels in IEC18 cells treated with LPS or probiotic supernatants at the indicated time periods. GAPDH was used as a loading control. (F) Densitometric analysis showing the ratios of LC3-II to LC3-I.
LPS alone (Fig. 3D-F). Our results showed that probiotic treatment decreased the number of autophagic vacuoles after LPS treatment, and hence, reduced the degree of autophagy activation.
Probiotics regulate the initiation and maturation of autophagy induced by LPS

The complete autophagy process can be divided into three stages: initiation, maturation, and degradation [30]. To further assess the biological significance of the autophagy pathway...
Fig. 5. Effects of *Bifidobacterium* on autophagy processes following LPS treatment. (A). IEC18 cells were treated with LPS and 50 nM chloroquine for 6 h and exposed to *Bifidobacterium* for 4 h. Western blot analysis of LC3 levels with GAPDH used as a loading control; (B). Densitometric analysis showing the ratios of LC3-II to LC3-I; (C). Probiotic-inhibited phagolysosomal degradation as indicated by inhibition of LPS-induced LAMP1 and CTSD expression as well as prevention of p62 degradation; (D). Ratios among LAMP1, CTSD, and p62 determined by densitometry analysis and normalized relative to GAPDH. Each bar represents the mean ± S.E. of three trials; (E). *ATG16L1* is essential for autophagy. Western blot analysis of *ATG16L1* protein showed successful down-regulation of *ATG16L1* induced by shRNA transfection; (F). Activation of LC3 following LPS or bacterial stimulation after transfection with negative shRNA or with shRNA; (G). Densitometric analysis showing the LC3-II/LC3-I ratio. Each bar represents the mean ± S.E. of three trials (** p < 0.01; * p < 0.05).
in response to LPS exposure, we transfected IEC18 cells with mCherry G-labeled LC3 expressed from a GFP-mCherry-LC3 tandem plasmid construct to follow autophagosome initiation and maturation. Confocal microscopy analysis showed that autophagy was not apparent following probiotic treatment alone (Fig. 4B) and that LPS stimulation resulted in a substantial (5-8 fold) increase in the number of cells containing GFP-mCherry-LC3 dots relative to control cells (Fig. 4C). When the transfected cells were treated with *B. bifidum* culture supernatants, the number of cells showing fluorescence signals markedly decreased to levels that were comparable to those for LPS treated cells (Fig. 4A, D, E). Collectively, these results demonstrate that in IEC-18 cells probiotics can regulate the initiation and maturation of autophagy associated with LPS stimulation.

**Probiotic treatment restored autophagic flux following chloroquine inhibition**

We next examined whether the elevated LC3-II protein level induced by LPS stimulation was due to decreased autophagic degradation or enhanced autophagosome formation. We first measured autophagic flux in the presence and absence of chloroquine (CQ), a known autophagosome-lysosome fusion inhibitor, following LPS treatment. CQ can raise lysosomal pH to suppress the activity of lysosomal acid hydrolases and thereby block the degradation of autophagic proteins that results in LC3-II accumulation [31]. IEC18 cells were treated with indicated doses of LPS and 50 nM chloroquine (Sigma) and analyzed by western blotting, which showed that exposure to LPS induced an approximately 4-fold increase in LC3-II levels in IEC18 cells, which was further increased to 6-fold in the presence of CQ (Fig. 5A, B). Interestingly, when chloroquine-treated cells were exposed to *B. bifidum* supernatants, the inhibition of autophagic flux was relieved as evidenced by a 5-fold reduction in LC3-II levels after probiotic treatment. To further examine the effect of probiotics on autophagic flux in LPS-treated cells, we next investigated the degradation stage of autophagy using two lysosome markers, lysosomal membrane-associated protein-1 (LAMP1), which is a essential to maintaining lysosomal function in the intestine, and cathepsin D (CTSD), a key acid hydrolase that localizes to the lysosome luminal space [32, 33]. Western blot analysis demonstrated that LAMP1 and CTSD protein levels were significantly up-regulated in the LPS-treated group compared to those in the untreated or probiotic stimulation group. Probiotic treatment inhibited LPS induced increases in LAMP1 and CTSD levels (Fig. 5C-D), suggesting that recruitment of these proteins to vesicles was inhibited, as was autophagosomal degradation. Another autophagy-associated protein, p62 (also known as sequestosome-1/SQSTM1), facilitates the degradation of polyubiquitinated proteins or organelles that in turn promotes its own degradation. Therefore, decreased p62 levels are indicative of autophagy activation and autophagic degradation [34]. IEC18 cells treated with LPS showed p62 activation that was needed to meet the increased demand for autophagosomes. However, probiotic treatment prevented p62 degradation, resulting in the accumulation of ubiquitinated p62 (Fig. 5C-D). Collectively, these results suggested that probiotics actually suppressed the maturation of autophagy induced by LPS treatment and could also inhibit phagolysosomal degradation.

**Downregulation of ATG16L1 expression does not influence probiotic-mediated effects on LPS-induced autophagy**

We next examined whether the probiotic-mediated suppression of LPS-induced autophagosome formation occurs through inhibition of autophagosome maturation during the initial stage of autophagy. We transfected IEC18 cells with shRNA designed to inhibit *ATG16L1* expression, which is required for autophagosome elongation [35] and thus *ATG16L1* knockdown would be expected to disturb the inhibitory effect of probiotics on LPS-induced autophagy. shRNA knockdown of *ATG16L1* expression effectively reduced LPS-mediated increases in *ATG16L1* protein levels and reduced expression by 64.8% relative to non-transfected cells (Fig. 5E). We subsequently examined autophagy of the shRNA-transfected cells in response to LPS and *B. bifidum* treatment using western blot analysis. Compared with control cells, knockdown of *ATG16L1* markedly reduced LC3-II protein expression.
activated by LPS treatment. However, the loss of LC3-II protein induced by *B. bifidum* exposure was not apparent in *ATG16L1* siRNA cells (Fig. 5F-G). In addition, we treated IEC18 cells with *B. bifidum* supernatants to assess effects on LPS-mediated changes in LC3 levels in the presence of *ATG16L1*-specific shRNA. Consistent with the inhibition observed with chloroquine treatment, LC3-II protein expression induced by LPS treatment of IEC18 cells decreased by ~2-fold (7.52 ± 0.74 to 3.53 ± 0.42 (p < 0.05)) in the presence of *ATG16L1* down-regulation (Fig. 5F-G). Taken together, these results showed that probiotic effects on LPS-induced autophagy appeared to occur throughout the autophagy process.

**Impaired physical barrier function following LPS treatment could be inhibited by probiotics**

For effective treatment of IBDs, restoring and sustaining physical barrier function of the gut epithelium is essential. Although probiotics are known to enhance intestinal barrier function [36], whether they also have positive effects on epithelial barrier dysfunction is unclear. Measurement of transepithelial electrical resistance (TEER) is one means to monitor intestinal barrier function. Of note, relative to other gut epithelia cell types, the IEC18 cells used in this study have lower baseline TEER values, which were consistent with those described in studies by Konsoula et al. [37] and Duizer et al. [38] (Fig. 6). The TEER values were slightly increased in the *B. bifidum* treated group, but they did not differ significantly from that of control cells. The addition of 5 μg/ml LPS resulted in a marked, time-dependent decline in TEER values. During the first 4 h after LPS exposure, TEER declined from 215.8 to 136.6 Ω·cm² and greater disruption in cell monolayers accompanied a decrease to 93.3 Ω·cm² after 6 h. To investigate the effect of probiotics on LPS-induced epithelial barrier dysfunction, TEER was measured 2 h or 4 h after *B. bifidum* supernatants were added into the apical and basal chamber of the inserts in LPS-exposed monolayers. *B. bifidum* treatment promoted significant recovery in TEER values (Fig. 6). Compared with cells treated with LPS alone for two hours, the TEER of the cells exposed to *B. bifidum* for 6 h increased by 30.8% (134.8 vs. 93.3 Ω·cm², P = 0.009) and by 41.3% (135.5 vs. 79.5 Ω·cm², P = 0.002) after 10 h, indicating that longer periods of probiotic exposure led to a gradual restoration of junction integrity. Additionally, compared with cells exposed to LPS for 4 h before the addition of probiotics, the TEER levels of probiotic-treated monolayers showed a significantly greater rise than that of monolayers incubated with LPS alone at 12 h (117.2 vs. 79.5 Ω·cm², P = 0.015). Taken together, our results suggested that exposure to *E. coli* LPS causes a rapid and dramatic loss of epithelial barrier integrity, which could be restored by treatment with probiotics.

**Discussion**

In the current study we report a novel role for probiotics in regulating autophagy induced by LPS. We demonstrated that LPS infection alone, but not probiotic treatment,
induced autophagy in intestinal cells. However, probiotic treatment attenuated LPS-induced autophagy and appeared to have effects during the initiation, maturation, and degradation stages of autophagy. Furthermore, our results showed that probiotics promoted a protective host response against intestinal barrier damage. Our study provides new insight into the critical role of probiotics in the pathogenesis of LPS exposure in intestinal epithelial cells.

Lipopolysaccharide (LPS) is a major outer membrane component of Gram-negative bacteria such as *E. coli*, and acts as a mediator of host responses that elicit intestinal endotoxicemia and multiple organ failure, as well as a stimulator of autophagic signaling in cultured intestinal epithelial cells and other cell types [39, 40]. Under normal physiological conditions, low levels of autophagy are maintained that play a vital role in host defenses against microbial infection [41]. Nonetheless, dysfunctions in the autophagy pathway have been linked to the pathogenic conditions involving autophagic cell death. Thus, we hypothesize that regulation of autophagic balance could be manipulated as a mechanism of LPS-related intestinal inflammation injury.

To elucidate if the molecular basis of probiotic-mediated protection against LPS induced injury is related to autophagy inhibition, we established an acute *in vitro* intestinal injury model using IEC18 rat epithelia cells and explored changes in expression of autophagy related proteins across several time points. A significant up-regulation of LC3-II protein expression and the ratio of LC3-II/LC3-I were observed after LPS administration to IEC18 cells, and this increase could be suppressed by the addition of probiotics after LPS treatment. Our results suggest that treatment with probiotics could effectively suppress increased autophagy that accompanies LPS-induced acute intestinal injury.

However, which autophagic stage probiotics affects is unclear. A more detailed characterization of the mechanisms by which probiotics regulate autophagy could yield new therapeutic strategies for IBD. To examine the different stages of autophagy and how they are affected by probiotics, we first inhibited LC3-II degradation by treating IEC18 cells with chloroquine and found a decrease in degradation after probiotic administration, as well as a parallel decrease in CTSD and LAMP1 levels. These results indicated that probiotic could influence autophagy at the phase when autophagosome degradation occurs. Then we asked whether probiotic could modulate autophagosomes at the initiation stage. We next took advantage of recent genome wide association studies that verified a SNP in the autophagy-related gene *ATG16L1* as a causal risk variant for Crohn's disease [13, 42]. Deficiencies in *ATG16L1* interfere with the recruitment of the *ATG12-ATG5* complex conjugate to the lysosomal membrane, resulting in a loss of LC3-I conjugation to phosphatidylethanolamine (PE) [35]. As such, *ATG16L1*-deficient cells cannot make LC3-II protein and autophagy is not induced. Here, *ATG16L1* shRNA-treated cells lacked LC3-II conversion in response to LPS, indicating that autophagy induction is required for *ATG16L1* activity, but we noted that in cells transfected with *ATG16L1* shRNA, probiotics still reduced the expression of autophagy markers to baseline levels after LPS intervention. Thus, these results indicate that *ATG16L1* likely does not play a major role in LPS-mediated autophagy. Taken together, the results suggest that probiotics might play a critical role in regulating the entire autophagy process, and that other mechanisms involving direct catalytic activity between LPS and probiotics might exist.

The intestinal epithelium is a single layer of columnar intestinal epithelial cells that comprises a physical and functional barrier to prevent invasion of undesirable antigens. Defects in epithelial barrier function have been demonstrated to be one of the major contributors to IBD pathogenesis [43]. Increases in intestinal permeability can facilitate the paracellular translocation of luminal antigens and stimulate underlying immune cells, thus leading to the chronic inflammation and exacerbation of colitis. As such, therapies that specifically restore tight junction and barrier function could have strong potential for treating IBD patients [44]. In this study, we employed beneficial microbes of the *Bifidobacterium* species to investigate the potential barrier effects on LPS-exposed IEC18 cells and found that *Bifidobacteria* could stabilize or even enhance the TEER of IEC18 cell monolayers, which
confirms the beneficial role of probiotics in protecting against intestinal epithelial barrier disruption induced by LPS.

In conclusion, we provided evidence that LPS could induce up-regulation of autophagy activity, while treatment with probiotics decreased autophagy and alleviated intestinal epithelial cell injury. Our results also emphasize that probiotics could play a significant role in regulating LPS-mediated autophagic activity in intestinal epithelial cells, and thus contribute to maintaining gut homeostasis. However, additional in vivo studies are needed to resolve the underlying mechanism by which probiotics inhibit LPS-induced autophagy, which would also provide insight into how probiotics regulate autophagy and modulate IBD pathogenesis. Together this information could be used to develop new therapeutic approaches for IBD patients.

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Disclosure Statement

All authors state that they have no conflict of interest.

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