Autophagy: A Novel Mechanism Involved in the Anti-Inflammatory Abilities of Probiotics

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Key Words
Probiotics • Inflammatory bowel diseases • Autophagy • Inflammation

Abstract
Background/Aims: Deregulation of the complex interaction among host genetics, gut microbiota and environmental factors on one hand and aberrant immune responses on the other hand, are known to be associated with the development of inflammatory bowel disease. Recent studies provided strong evidence that autophagy plays a key role in the etiology of Crohn’s disease (CD). Probiotics may exhibit many therapeutic properties, including anti-inflammatory abilities. While successful results have been obtained in ulcerative colitis patients, probiotics remain inefficient in CD for unknown reason. It remains therefore important to better understand their molecular mechanisms of action. Methods: The activation of autophagy was examined by stimulating bone marrow-derived dendritic cells by the bacteria, followed by confocal microscopy and western blot analysis. The impact of blocking in vitro autophagy was performed in peripheral blood mononuclear cells using 3-methyl adenine or bafilomycin followed by cytokine secretion measurement by ELISA. The role of autophagy in the anti-inflammatory capacities of the bacterial strains was evaluated in vivo using an acute trinitrobenzene sulfonic acid-induced murine model of colitis. The impact of BMDC was evaluated by adoptive transfer, notably using bone marrow cells derived from autophagy-related 16-like 1-deficient mice. Results: We showed that selected lactobacilli and bifidobacteria are able to induce autophagy activation in BMDCs. Blocking in vitro autophagy abolished the capacity of the strains to induce the release of the anti-inflammatory cytokine
interleukin-10, while it exacerbated the secretion of the pro-inflammatory cytokine interleukin-1β. We confirmed in the TNBS-induced mouse model of colitis that autophagy is involved in the protective capacity of these selected strains, and showed that dendritic cells are involved in this process. **Conclusion:** We propose autophagy as a novel mechanism involved in the regulatory capacities of probiotics.

**Introduction**

Inflammatory bowel disease (IBD), encompassing the main clinical forms Crohn’s disease (CD) and ulcerative colitis (UC), is characterized by chronic inflammation of the gut mucosa, disruption of the gut barrier function and a dysbiotic microbiota [1]. Indeed, the gut microbiota of IBD patients is characterized by a low microbial diversity, a reduced abundance of bifidobacteria, lactobacilli [2] and *Faecalibacterium prausnitzii* [3], and a higher abundance of pathobionts such as adherent-invasive *Escherichia coli* [4]. Moreover, abnormal immune responses are elicited against the luminal bacteria in genetically susceptible individuals [5]. Genome-wide association studies (GWAS) have contributed to the identification of more than 200 genetic risk loci for IBD [6]. Many of these genes are related to innate immunity, mucosal barrier function and bacterial recognition. Notably, the gene encoding the nucleotide-binding oligomerization domain 2 (NOD2) receptor, which senses muramyl dipeptides, the conserved motif of the peptidoglycan of both gram-negative and gram-positive bacteria, has been largely shown as the main susceptibility gene associated with CD [7]. Different polymorphisms of genes involved in autophagy, such as *atg16l1* (autophagy-related 16-like 1) [8], *IRGM* (immunity related guanosine triphosphatase) [9] and *LRRK2* (leucine-rich repeat kinase 2) have also been reported [10].

Autophagy is an intracellular degradation pathway that regulates the turnover of cellular proteins and organelles having essential roles in cellular homeostasis [11]. The autophagic process starts with the formation of the phagophore, a double-membrane compartment which engulfs the cargo to be degraded and closes off to form the autophagosome that finally leads to fusion lysosomes and the degradation of the cargo by the lysosomal hydrolases. This process is highly complex and orchestrated by numerous proteins, including more than 40 autophagy-related proteins (ATG) [12]. The formation of the autophagosome is regulated mainly by the ATG5–ATG12 complex, then stabilized by ATG16L1, which allows the modification of the microtubule-associated light chain 3 (LC3), initially present in the cytoplasm, to a phosphatidylethanolamine (PE)-conjugated membrane-bound form (LC3-II), which is finally recruited into the autophagosomes [13]. The engulfment of the cargo, closure of the autophagosome and fusion with the lysosomal compartment is orchestrated by LC3, which is widely used as a marker of autophagy activation [14]. In the gut, autophagy has been reported to mediate crucial functions in innate and adaptive immunity, such as antigen presentation by dendritic cells (DC) [15], cytokine secretion by macrophages [16] and antimicrobial peptide secretion by Paneth cells [17]. Recently, a critical role for autophagy in regulating survival of intestinal epithelial cells in response to TNF-α [18] as well as an unexpected critical impact in coordinating the pro-regenerative IL-22 signaling [19] have been reported. Autophagy plays also an essential role during infection by degrading intracellular pathogens through xenophagy [20]. Interestingly, NOD2 is known to be involved in the autophagy process, since it is able to recruit ATG16L1 into the plasma membrane, at the bacterial entry site [21]. As such, NOD2 and ATG16L1 deficiencies have been shown to affect bacterial clearance *in vitro*, favoring the survival of intracellular bacteria, and explaining the proliferation of certain pathobionts, and observed pro-inflammatory cytokine secretions [22, 23]. It has been reported that autophagic flux is impaired in the damaged mucosa of IBD patients, as compared to non-damaged tissue, suggesting that, in addition to genetic components, inflammation regulates this process [24]. In parallel, strong evidence indicates that autophagy regulates inflammatory responses, notably by controlling the inflammasome activity, explaining that compromised autophagy...
causes aberrant inflammatory responses, which can lead to the development of IBD [25]. Deregulation of DC function has been reported in DCs from CD patients carrying NOD2 or ATG16L1 risk variants, which are defective in autophagy induction, bacterial trafficking and antigen presentation [22]. Disruption of atg16l1 in CD11c+ DCs significantly increased the severity of the inflammation induced by dextran sodium sulfate (DSS) [26]. Recently, an inhibitory role of autophagy in the immunogenic maturation of DCs and a positive role in their tolerogenic maturation was suggested [27]. Finally, Kabat et al. recently showed that autophagy defects can alter the balance of different types of T cells in the gut, leading to intestinal inflammation. By specific ablation of atg16l1 in FoxP3+ T-regulatory cells in mice, they demonstrated that autophagy promotes the survival of these cells in the gut [28]. The autophagy machinery in IBD represents therefore a complex pathway that contributes to the pathogenesis of the disease.

Despite all advances of targeted therapies including biological treatments, which have substantially improved the patient’s quality of life, still many problems remain unsolved with notably adverse health consequences. The development of alternative therapies using natural products is therefore gaining worldwide attention [29]. Targeting the gut bacteria dysbiosis and impaired gut functions linked to IBD may constitute a target of choice in the treatment of chronic inflammatory diseases. In this context, manipulation of the gut ecosystem using beneficial microbes, namely probiotics, has been a major focus of research during the last decade. However, their recommendation for IBD treatment is still rather scarce. In order to select more effective strains, it is thus important to better define the underlying mechanism of action and screen for targeted functional properties. We previously showed that the anti-inflammatory capacities of lactobacilli and bifidobacteria are strain-specific and that their in vitro immunomodulatory capacities strongly correlate with their protective capacities in experimental colitis models [30, 31]. Moreover, we highlighted the crucial role of DCs in this probiotic functionality [32]. In the present study, we evaluated both in vitro and in vivo the role of autophagy in the immunoregulatory capacities of selected probiotic strains.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains evaluated in the present study are listed in Table 1. Lactobacilli were grown in de Man Rogosa and Sharpe broth (MRS, Difco, Detroit, USA) overnight at 37 °C under microaerophilic conditions. Bifidobacteria were cultured overnight in MRS broth, supplemented with 0.1% (w/v) L-cysteine hydrochloride (Sigma, USA) under anaerobic conditions using anaerobic generator packs (GENbaganaer, Biomérieux, France). For in vitro experiments, bacteria were washed twice with sterile phosphate buffered saline (PBS, pH 7.2), and aliquoted at 10^9 CFU/ml in PBS supplemented with 20% (v/v) glycerol and stored at -80 °C. For in vivo studies, freshly grown bacteria were adjusted to 5 × 10^9 CFU/ml in gavage buffer (200 mM NaHCO3 containing 1% glucose).

In vitro stimulation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from human blood obtained as described previously, by authorized staff, from five healthy donors upon approved agreement (signed consent) [31]. Briefly, after a Ficoll gradient centrifugation (Pharmacia, Stockholm, Sweden), mononuclear cells were collected, washed twice with PBS and adjusted to 2 x 10^6 cells/ml in RPMI 1640 (Roswell Park Memorial Institute, Gibco, Scotland), supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, Scotland), 1 mM glutamine and 150 μg/ml gentamycin. Cells (10^6 cells/well) were plated in 48-well cell culture plates and were pre-incubated for 1 hour at 37 °C in the presence or not of 3-methyl adenine (3-MA, Sigma, 5 mM)

<p>| Table 1. List of bacterial strains used in the study |</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Source of isolation</th>
</tr>
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<tbody>
<tr>
<td>B. bifidum</td>
<td>IPL A7.31</td>
<td>Infant stool</td>
</tr>
<tr>
<td>B. longum</td>
<td>CMUL CLX001</td>
<td>Human vagina</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>100-23</td>
<td>Rodent intestine [64]</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>IPL A6.33</td>
<td>Human vagina</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>IPL A2.21</td>
<td>Human vagina</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>CMUL057</td>
<td>Human vagina</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>CMUL067</td>
<td>Human vagina</td>
</tr>
</tbody>
</table>
or bafilomycin A1 (Sigma, 100 nM). PBMCs were subsequently stimulated with bacteria at a bacteria/cell ratio of 10:1. PBS with 20% glycerol was used as negative control. After 20 h of stimulation at 37 °C under 5% CO₂, supernatants were collected, clarified by centrifugation and stored at -20 °C for cytokine analysis. The cytokines IL-10 and IL-1β were measured by ELISA using the ELISA MAX™ standard set (Biologend, San Diego, USA) and the Duoset kit (R&D System, USA), respectively, according to the manufacturer’s instructions. The impact of 3-MA on the autophagy activation induced by the bacterial strains was controlled by western blot, confirming the inhibitory effect of 3-MA (Fig. S1 – for all supplemental material see www.cellphysiolbiochem.com). Bafilomycin is known to induce LC3-II accumulation by blocking the autophagic flux and preventing the acidification of endosomes [33]. For that reason we did not check the impact of LC3 conversion by western blot after blockade with bafilomycin.

**Bone marrow-derived dendritic cell preparation (BMDCs)**

BMDCs were generated from the bone marrow precursors isolated from femurs and tibias of BALB/c mice as described by Lutz et al. [34], with minor modifications. Briefly, after red cell lysis using 0.142 M NH₄Cl / 0.04X PBS buffer, the cells were grown at 2 x 10⁶ cells in 10 ml of IMDM medium (Isclose’s Modified Dulbecco’s Medium, Gibco) supplemented with 10% inactivated FCS (Fetal Calf Serum, Gibco, Scotland), 50 μM β-mercaptoethanol, 1 mM glutamine, 50 μg/ml gentamycin, and 10% of supernatant from a granulocyte macrophage colony-stimulating factor-expressing cell line (GMCSF transfected J588 myeloma cell line). Freshly prepared medium was added every three days. BMDCs were used between day 10 and 13 of culture (maximum of CD11c expression as checked by FACS analysis).

**BMDC stimulation**

BMDCs (0.75 x 10⁶ cells/well) were plated in 24-well cell culture plates and were pre-incubated for 1 h at 37 °C in the presence or not of 3-MA (Sigma, 5 mM). After the pre-treatment, BMDCs were stimulated with bacteria at a bacteria/cell ratio of 10:1 in the presence of 150 μg/ml gentamycin. After 20 h, culture supernatants were collected, clarified by centrifugation and stored at -20 °C for IL-1β measurement by ELISA using the Duoset kits (R&D System, USA), according to the manufacturer’s instructions.

For in vivo cell transfer, the cells were grown at 5 x 10⁶ cells in a Petri dish in 10 ml of complete IMDM and were pre-incubated for 1 hour at 37 °C in the presence or not of 3-MA (Sigma, 5 mM). BMDCs were then stimulated with bacteria at a bacteria/cell ratio of 10:1 in the presence of 150 μg/ml gentamycin, washed 3 times in PBS and resuspended at 2 x 10⁶ cells in 200 μl of PBS. No remaining viable bacteria were detected in the cell suspensions, as checked by plating on MRS medium.

Trypan blue staining was used to count the number of viable BMDCs. No significant difference (P=NS) was obtained between untreated WT BMDCs (11.2 ± 3.1 % mortality) and cells treated with 3-MA (15.9 ± 3.3% mortality).

**Western-Blot analysis**

BMDCs were plated in 24-well cell culture plates at 1 x 10⁶ cells/well and were pre-incubated for 1 h at 37 °C in the presence or not of bafilomycin A1 (Sigma, 100 nM) or 3-MA (5 mM). BMDCs were then stimulated with the bacteria at a bacteria/cell ratio of 10:1 for 4 h. PBS with 20% glycerol was used as negative control and rapamycin (10 μg/ml) as positive control. The cells were washed with PBS and then lysed with a lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA pH 8, 1% of Triton 100X and 0.1% SDS), supplemented with a protein inhibitor (Protease inhibitor cocktail tablets, Roche, Mannheim, Germany) according to the manufacturer’s instructions. The cell lysates were harvested and centrifuged at 12,000 x g for 15 min at 4 °C. The supernatants were recovered and mixed with loading buffer (Laemmli buffer) containing 60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue and then heated for 15 min at 75 °C. The migration was carried out for 2 h in a 15% acryl/bis acryl gel in 0.025 M Tris buffer / 0.192 M glycine / 0.1% SDS at 90 V. After migration, the proteins were transferred to a PVDF membrane (Polyvinylidene Fluoride, Hybond-p; Bio-Rad, Hercules CA, USA) in a 20 mM Tris / 150 mM glycine / 20% methanol buffer for 2 h at 100 V. Membranes were treated with blocking buffer containing 10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20 and 3% BSA (Bovine Serum Albumin) for 1 h at 25 °C and incubated overnight at 4 °C with a rabbit anti-LC3 (Novus, 1:3000, Littleton, CO, USA) or mouse anti-α-tubulin antibody (Invitrogen, 1:5000, Carlsbad, CA USA) in blocking buffer. After washing for 15 min in PBS / 0.01% Tween, they were incubated for 45 min at 25 °C with a secondary anti-mouse or anti-
rabbit IgG conjugated with HRP (Promega, 1:5000, Madison, WI, USA) in PBS/Tween 0.01%. Membranes were washed as described and incubated with ECL detection reagent (ECL Plus, Western blotting reagent, Amersham, GE Healthcare, Buckinghamshire, UK). The light emitted following the chemiluminescence reaction was detected and analyzed by the Image LAS-3000 system (Fujifilm, Japan) and the band intensity was quantified using the Multi Gauge V3.0 software (Fujifilm, Japan).

**Immunofluorescence and confocal microscopy analysis**

BMDCs were grown on coverslips in 24-well plates at 0.2 x 10⁶ cells/well. They were stimulated as described above and fixed with Paraformaldehyde (4% PFA in PBS) for 15 min. After washing with PBS, the cells were permeabilized with 10% TRITON X 100 solution for 5 min and blocked for 30 min with pure fetal calf serum. The cells were then incubated for 1 hour with anti-mouse LC3 primary antibody (Anti-LC3 PM036, MBL, 1:100) in fetal calf serum. After 3 washes for 5 min in PBS, the coverslips were incubated for 45 minutes with the fluorochrome-coupled anti-rabbit secondary antibody (Alexa fluor 488 conjugated antibody, Invitrogen, 1:500) in FCS. After 3 washings in PBS, nuclei were labeled with DAPI (4',6'-diamidino-2-phenylindole, DNA marker emitting at 359/461 nm, Invitrogen, 1:1000) in PBS for 5 min. Cells were then washed with PBS and the slides were mounted on glass slides using 7 μl of Dako Fluorescence Mounting Medium (Carpinteria, CA, USA). Confocal microscopy was performed using a confocal laser scanning microscope (Zeiss, LSM 880).

**Experimental TNBS-induced colitis model and study design**

Female BALB/c ByJ mice (7-8 weeks old) were purchased from Janvier Laboratories (Le Genest-Saint-Ise, France). Before experimentation, animals were provided a one week acclimation period. Mice were randomly divided and maintained under specific pathogen-free conditions in a controlled environment (temperature of 22 °C, 12 h/12 h light/dark cycle and with ad libitum access to food and water).

Protective capacities of probiotics were evaluated in vivo using a standardized murine model of acute colitis, induced by the administration of TNBS [31]. Mice (n=10 per group) received daily live bacteria by intra-gastric administration (5 x 10⁸ CFU/day/mice), in 100 µl gavage buffer (0.2M NaHCO₃, 1 % glucose), or gavage buffer alone (control healthy mice or TNBS-treated control mice) for six consecutive days before colitis induction and 1 day after the TNBS administration. The blockage of autophagy was performed by intraperitoneal administration of the 3-MA inhibitor (Sigma, 10 mg/kg) concomitantly with the bacterial treatment (day 1 to day 6). At day 5, anesthetized BALB/c mice were intra-rectally administered with 50 µl of a solution of TNBS (Sigma-Aldrich, France) dissolved in 0.9% NaCl/ethanol solution (50/50 v/v), at a dose of 105 mg/kg, while control healthy mice received only 50% ethanol. The mice were sacrificed 48 h after the induction of colitis. Macroscopic inflammatory scores were determined blindly according to the Wallace scoring method [35] by assessing the intensity of inflammation and the extent of lesions (thickening of the intestinal wall, intensity ulceration and colonic necrosis). Blood was collected by retro-orbital sampling and the plasma was recovered by centrifugation and stored at -80 °C until RNA extraction and real-time RT-PCR analysis.

The protective effect of untreated or *L. rhamnosus* IPL A21.1-pulsed BMDCs in the presence or absence of 3-MA was also evaluated in BALB/c mice by intra-peritoneal administration of 2 x 10⁶ cells, 2 h before TNBS-colitis induction as previously described [32] and study was performed as described above.

To evaluate the role of autophagy of the *L. rhamnosus*-pulsed DCs, we also used BMDCs derived from mice having *Atg16l1* deficiency, specifically in myeloid cells (*atg16l1lox/lox-CreLys*). BMDCs derived from WT C57BL/6 Jr mice or *atg16l1lox/lox* mice were used as controls. BMDCs were obtained, stimulated and transferred as described above for those derived from BALB/c mice. Trypan blue staining was used to count the number of viable cells and no significant difference (P=NS) was obtained between untreated WT BMDCs.
(11.2 ± 3.1 % mortality) and atg16l1-deficient cells (21.5 ± 6.8% mortality). Colitis was induced in C57BL/6 Jr using TNBS at a dose of 150 mg/kg.

The impact of 3-MA on the autophagy activation induced by the bacterial strains or rapamycin and the activation in atg16l1-deficient-cells was controlled by western blot, confirming the inhibitory effect of 3-MA and the absence of activation in deficient BMDCs (Fig. S2). As mentioned for PBMCs, we did not check the impact of LC3 conversion after blockade with bafilomycin known to induce LC3-II accumulation.

RNA extraction and qRT-PCR

Tissue samples were homogenized using Lysing Matrix D (MP Bio, Solon, OH, USA). Total RNA was extracted using Macherey-Nagel NucleoSpin RNAII isolation kit according to the manufacturer’s recommendation (Macherey-Nagel, Germany). Quantity and quality of RNA were checked using nanodrop (260/280 nm, 260/230 nm), showing a purity ratio 260/280 higher than 1.95 in all samples. Reverse transcription of total RNA (1 μg) was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems™, Foster City, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed using SYBR green as dye (Applied Biosystems™). Briefly 2.5 μl of cDNA (corresponding to 25 ng of RNA) was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems™) in the presence of 200 nM specific primers in a final volume of 10 μl using the QuantStudio® 3D Digital PCR System (Applied BiosystemsTM). Relative mRNA levels \[2^{\Delta\Delta \text{CT}}\] were determined by comparing (i) the PCR cycle thresholds (Ct) for the gene of interest and the housekeeping gene Actb (ΔCt) and (ii) the ΔCt values for treated and untreated animal groups (ΔΔCt).

Statistical analysis

GraphPad Prism was employed for graph preparation and statistical evaluation. Differences between groups were assessed using ANOVA, followed by nonparametric Mann-Whitney test. Data with p values ≤ 0.05 were considered to be significant.

Statement of ethics

Animal experiments were performed at the animal facility of the Institute Pasteur of Lille (number A59107, Lille, France) and carried out in accordance with the guidelines of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (number 86/609/EEC). All the studies were approved by the local investigational ethics review board (Nord-Pas-de-Calais CEEA N°75, Lille, France) and the Ministère de l’Éducation Nationale, de l’Enseignement Supérieur et de la Recherche, France (accredited number 201608251651940). The animal experiments also complied with French legislation (Government Act 87-848) and the European Communities Amendment of Cruelty to Animals Act 1976.

Results

Bacterial strains induced autophagy in BMDC

We first evaluated the ability of selected strains to activate the autophagy process after stimulating BMDCs from BALB/c mice with the different bacterial strains. LC3 was used as a quantitative marker of autophagy since it is required for the formation of the autophagosome in the cell and its accumulation is proportional to the amount of autophagosomes. Immuno-labelling followed by confocal laser scanning analysis allowed to determine the number of autophagosomes in the form of LC3-labelled fluorescent punctate structures. The transformation of LC3 from the cytosolic form (LC3-I) to the lipidated form (LC3-II) during the autophagy process was also followed by western blot [37].

As shown in Fig. 1A, treatment of cells with the autophagy activator rapamycin for 4 h significantly (p < 0.001) increased LC3 puncta. Most of the strains were able to induce significant formation of autophagosomes, the activation being higher after Lactobacillus gasseri IPL A6.33 and Bifidobacterium longum CMUL CXL 001 (p < 0.001) stimulation, while Lactobacillus reuteri 100-23 did not activate this process in this condition (p=0.497). This was confirmed by measuring the ratio of the levels of LC3-II to LC3-I after western blotting.
analysis. As showed in Fig. 1B, significantly increased LC3-II/LC3-I levels was observed in cells treated with rapamycin, as compared to the basal level observed for untreated cells (p < 0.01). A slight basal activation could also be detected with the strains. This activation was significant for *Bifidobacterium bifidum* IPL A7.31 and *L. gasseri* IPL A6.33 (p < 0.05), and was increased when cells were pre-treated with bafilomycin, a potent inhibitor of the lysosomal proton pump V-ATPases which inhibits autophagic flux by preventing the acidification of endosomes and lysosomes [33]. This shows that the potential probiotic strains increase LC3-II level by activating autophagy but not by blocking autophagosome-lysosome fusion.

Inhibition of autophagy enhanced IL-1β production and decreased IL-10 secretion in human immune cells and murine BMDCs stimulated with bacteria

In order to evaluate the role of autophagy in the immuno-modulation capacities of these selected bacteria, we used two different pharmacological inhibitors: the 3-MA, a PI3K inhibitor which blocks the autophagosome formation [38] and bafilomycin A1. PBMCs were pre-treated or not with the inhibitors for 1 h and subsequently stimulated by the different strains for 20 h, and secreted cytokine amount was measured. All the tested strains induced high levels of IL-10 (Fig. 2A), the highest levels being obtained with *Lactobacillus*
rhamnosus IPL A2.21, L. gasseri IPL A6.33 and L. reuteri 100-23, and somewhat lower levels for L. gasseri CMUL057 and Lactobacillus acidophilus CMUL067 as previously observed [39]. The capacity of the strains to induce IL-1β was strain-dependent and was inversely correlated with their respective IL-10-inducing capacity with L. gasseri CMUL057 and L. acidophilus CMUL067 being the highest inducers. The two autophagy inhibitors were able to significantly (p < 0.05 or 0.01, respectively) limit the IL-10 production induced by all the strains, with a more pronounced effect for bafilomycin treatment, which completely blocked IL-10 induction. Conversely, the bacteria-mediated induction of IL-1β was significantly enhanced when PBMCs were pre-treated with both inhibitors. This effect was also greater with bafilomycin, especially for the strains that induced moderate basal levels of IL-1β (p < 0.05 or 0.01, respectively). The role of autophagy in the immunomodulatory capacity of the bacterial strains was also confirmed using murine BMDCs. Since low amounts of IL-10 were induced after bacterial stimulation, it was not possible to highlight an effect in the presence of inhibitors (data not shown), however, the blockage of autophagy using 3-MA or the use of atg16l1-deficient cells having myeloid cell-specific Atg16l1 deficiency (atg16l1fl/−/CreLyz), increased probiotic-induced secretion of IL-1β as also observed on PBMCs (Fig. 2B and 2C).

Fig. 2. Blockage of autophagy enhanced IL-1β production while decreased IL-10 secretion in human PBMCs and murine BMDCs stimulated by the bacteria. (A) PBMCs (2 x 10^6 cells/ml, n = 5 different donors) were pre-treated or not for 1 h at 37 °C with 3-MA (5 mM) or bafilomycin (100 nM) and subsequently stimulated by the different strains for 20 h. IL-10 and IL-1β release was measured in the supernatants. Data represent means ± SEM of 5 independent donors. * refers to the comparison of bacteria-stimulated PBMCs in the presence or absence of the inhibitors; *p<0.05, **p<0.01 (B) WT BMDCs pre-treated or not for 1 h at 37 °C with 3-MA (5 mM) or (C) WT and atg16l1-deficient (KO) BMDCs (0.75 x 10^6 cells/well) were stimulated by the different strains for 20 h. IL-1β release was measured in the supernatants. Data represent means ± SEM of 3 different experiments. *p<0.05 refers to the comparison of bacteria-stimulated BMDCs in the presence or absence of 3-MA.
In vivo inhibition of autophagy limited the protective effects of selected strains in an acute TNBS-induced murine model of colitis

We evaluated the role of the autophagy process in the protective capacity of potential probiotic strains using a well-established model of acute colitis, induced by TNBS (Fig. 3A) in BALB/c mice. We selected on the one hand *L. rhamnosus* A2.21, which previously was shown to protect through an induction of immune-regulatory response and had a capacity to restore the epithelial barrier, and *L. acidophilus* CMUL067 on the other hand, which exhibited its protective effect essentially through an important strengthening of the epithelial barrier [39]. Bacteria were administrated daily by oral gavage and the blockage of autophagy was performed by intraperitoneal (IP) injection of 3-MA, concomitantly with the bacterial administration. As expected, TNBS induced a strong inflammation, depicted by an average weight loss of 16.7% (Fig. 3B), highly significant macroscopic (Wallace score of 7.3, p < 0.01) and histological scores (Ameho score of 5.6, p < 0.01) and a strong increase of the IL-6 plasmatic concentration (p < 0.01) (Fig. 3B-F), as compared to untreated control animals. This was further confirmed by strong colonic mRNA expression levels of the pro-inflammatory cytokines IL-6 and TNF-α and the chemokine CXCL2 (p < 0.01) 48 h after colitis induction (Fig. 3G), compared to untreated control mice.

The two selected strains were able to dampen the acute colitis, resulting in significant decreases of weight loss and both macroscopic (p < 0.001) and histological scores of inflammation (p < 0.05) (Fig. 3B, C and E), in comparison to mice receiving buffer only (TNBS control group). The protective ability of the strains was further confirmed by a significant decrease in the level of plasmatic IL-6 (p < 0.01) and in the expression of pro-inflammatory genes (p < 0.05 or 0.01) (Fig. 3F-G). In mice receiving only TNBS as well as the *L. acidophilus* strain, administration of 3-MA did not significantly reduce the loss of body weight, the macroscopic and histological scores of inflammation, nor the plasmatic IL-6 concentration (Fig. 3B, C and E), while its significantly (p < 0.05 or 0.01) limited the protective effects induced by the *L. rhamnosus* A2.21 strain for all the parameters tested. This observation suggested that the blockage of the autophagy process prevented the anti-inflammatory effects induced by the *L. rhamnosus* strain. Interestingly, the daily injection of 3-MA led to an increased level of plasmatic IL-1β in all groups of mice, even with the animals that received the protective *L. rhamnosus* IPL A2.21 strain (Fig. 3F).

While TNBS treatment induced a significant decrease in zo-1 and occludin mRNA expression levels (p < 0.01) in control mice, the administration of selected strains could significantly restore expression of the genes encoding these tight junction (TJ) proteins (Fig. 3G). In comparison to control TNBS-treated mice, a higher expression level was obtained with *L. acidophilus*, as reported previously [39]. The administration of 3-MA did not significantly affect the capacity of the strains to induce the expression of TJ genes.

The protective role of probiotic-treated BMDC is abolished after pharmacological inhibition of autophagy or using atg16l1-deficient cells

We previously demonstrated that the adoptive transfer of BMDCs pulsed in vitro with selected lactobacilli exhibited in vivo protection against colitis in mice [32]. In the present study, we confirmed that a single IP administration of BMDCs pulsed with *L. rhamnosus* IPL A2.21 rescued BALB/c mice from TNBS-induced colitis (105 mg TNBS/kg), while untreated DCs had no effect (Fig. 4). The protective ability of bacteria-treated BMDCs was characterized by a significant reduction of the weight loss (p < 0.01), decrease in macroscopic and histological scores of inflammation (p < 0.01 and p < 0.001, respectively) and a strong decrease of plasmatic IL-6 secretion (p < 0.01), while these parameters were not significantly changed in mice receiving untreated BMDCs (Fig. 4B-E). This was confirmed by measuring *il6* and *cxcl2* mRNA expression levels (Fig. 4G). We therefore evaluated the role of autophagy in the immune-regulatory capacities of *L. rhamnosus*-treated DCs by stimulating the BMDCs with the bacteria in the presence or absence of 3-MA before the adoptive transfer. *L. rhamnosus*-pulsed DCs, in the presence of 3-MA, were no longer able to protect mice from colitis. Body weight loss, macroscopic and histological scores of inflammation, plasmatic...
**Fig. 3.** In vivo inhibition of autophagy limited the capacity of *L. rhamnosus* A2.21 to rescue mice from acute TNBS-induced colitis. (A) Experimental protocol used for the mouse model of acute colitis in BALB/c mice was induced by intrarectal administration of TNBS (105 mg/Kg), intragastric administration of bacteria and IP injection of 3-MA. (B) Body weight loss (as a percentage of the initial weight) (C) Macroscopic evaluation of colonic inflammation (Wallace score) (D) Representative histological sections (stained by May Grünwald Giemsa, 100X magnification) of mice treated (TNBS) or not (Healthy mice) with TNBS, and orally administrated or not with the selected strains and treated or not by IP injection of 3-MA (E) Histologic evaluation of colonic inflammation (Ameho score) (F) Plasmatic IL-6 and IL-1β concentrations measured by ELISA, two days after colitis induction (G) Gene expression of cxcl2, il6, tnfa, zo1, and occludin from colonic samples. Values are expressed as the relative mRNA levels of samples compared with colons from healthy mice. The data represent the mean values of each group (n=10) ± SEM. * and # refer to the comparison of bacteria-treated groups versus TNBS control group or TNBS group versus healthy mice, respectively; *p<0.05, **or***p<0.01, ***p<0.001.
Fig. 4. The protective capacity of the adoptive transfer of *L. rhamnosus*-treated BMDC is abolished after pharmacological inhibition of autophagy. (A) Experimental protocol used for the murine model of acute colitis in BALB/c mice induced by intrarectal administration of TNBS (105 mg/Kg) and adoptive transfer of BMDCs (B) Body weight loss (as a percentage of the initial weight) (C) Macroscopic evaluation of colonic inflammation (Wallace score) (D) Histologic evaluation of colonic inflammation (Ameho score) (E) Representative histological sections (stained by May Grünwald Giemsa, 100X magnification) of mice treated (TNBS) or not (Healthy mice) with TNBS, and IP treated with *L. rhamnosus*-pulsed or not BMDCs, treated or not with 3-MA (F) Plasma IL-6 concentrations, measured two days after colitis induction by ELISA (G) Gene expression of *cxcl2, il6, tnfa*, and *il1b* from colonic samples. Values are expressed as the relative mRNA levels of samples compared with colons from healthy mice. The data represent the mean values of each group (n=10) ± SEM. * and # refer to the comparison of BMDCs-treated groups versus TNBS control group or TNBS group versus healthy mice, respectively; * or *p<0.05, ** or "p<0.01, ***p<0.001.
IL-6 levels and mRNA expression levels of pro-inflammatory cytokines and chemokines were not significantly different from those obtained from the control TNBS-treated group or the group receiving the BMDCs treated with 3-MA alone (Fig. 4B-G).

Similar results were obtained for C57BL/6 mice (Fig. 5), in which TNBS treatment (150 mg/kg) induced severe colitis, resulting in an average weight loss of 14.93% (p < 0.001), a Wallace score of 6.07 ± 0.81 (p < 0.001) and strong colonic mRNA expression levels of the pro-inflammatory genes encoding CXCL2, TNF-α, IL-1β and IL-17, compared to untreated control mice (p < 0.001). The mice that received the DCs derived from wild-type (WT) and control (atg16l1flox/flox) mice or mice having myeloid cell-specific Atg16l1 deficiency (atg16l1flox/floxCreLyz), which were not pulsed by the bacteria, did not show a significant difference compared with the control group treated with TNBS only. As obtained in the BALB/c mouse model, the adoptive transfer of *L. rhamnosus* A2.21-pulsed WT and control BMDCs led to a significant protection, as shown by the reduction of the Wallace score (p < 0.01), compared to controls. Fig. 5. The protective capacity of the adoptive transfer of *L. rhamnosus*-treated BMDCs is abolished in atg16l1-deficient cells (A) Body weight loss (as a percentage of the initial weight) (B) Macroscopic evaluation of colonic inflammation (Wallace score) (C) Gene expression of cxcl2, il6, tnfα, il1b and il17 from colonic samples. Values are expressed as the relative mRNA levels of samples compared with colons from healthy mice. The data represent the mean values of each group (n=10) ± SEM. * and # refer to the comparison of BMDCs-treated groups versus TNBS control group or TNBS group versus healthy mice, respectively; *p<0.05, **or ## p<0.01, ### p<0.001.
to transferring *L. rhamnous* A2.21-pulsed Atg16l1-deficient BMDCs. This was confirmed by measuring the weight loss and the expression of pro-inflammatory genes.

**Discussion**

Inflammatory bowel disease (IBD) is a chronic immune disorder of unclear etiology, characterized by disruption of the epithelial barrier function and by chronic inflammation of the mucosa [1]. Multiple factors play a role in the pathogenesis of the disease, including genetic susceptibility, immunological and environmental factors and modification of the gut microbiota. GWAS have revealed numerous polymorphisms in autophagy-associated genes, providing growing evidence for a defective autophagy process in the pathogenesis of IBD, particularly in CD patients. Impaired autophagy has been observed in the damaged mucosa of IBD patients and in the mucosa of TNBS-treated mice [24]. Despite significant advances, current therapies for IBD remain inefficient or come with unacceptable long-term side effects and they predominantly target pathological immune responses rather than the potential causal factors. Given the growing interest in the role of autophagy in regulating gut homeostasis [40], it has become an attractive target for exploring the impact of therapeutic approaches that address the autophagy process. Promising results have been obtained by the administration of sirolimus (rapamycin) which led to a sustained improvement of disease symptoms in refractory CD patients, including reduced inflammation and improved endoscopic appearance [41]. Stimulation of autophagy using different pharmacological agents, such as rapamycin, betanin or trehalose, has also been shown to prevent the impaired autophagic flux induced by TNBS and to ameliorate murine colitis by inhibiting inflammation [42]. However, current therapies are often associated with adverse side effects. As a consequence, research attention is now focusing on alternative therapies, preferably based on natural and safe products. The capacity of probiotics to improve gut health has received considerable scientific interests for more than a century, and accumulating evidence has also supported their positive impact in the control of gut inflammation. Probiotics can exhibit their protective effects through different mechanisms, notably the modulation of the gut microbiota, the improvement of the gut barrier function and the modulation of the mucosal immune system, protecting the intestinal mucosa from pathogens. In the present study, we investigated the possibility that activation of autophagy might be involved in the anti-inflammatory capacities of selected lactobacilli and bifidobacteria that we previously highlighted to be protective in a TNBS-induced murine model of colitis [39].

The detection of LC3-labelled puncta by confocal microscopy allowed us to demonstrate that the selected strains were able to activate autophagy, except the *L. reuteri* strain. This was confirmed using western blots measuring the conversion of LC3I to LC3II. Notably the use of bafilomycin, which blocks the fusion between autophagosomes and lysosomes, allowed us to confirm autophagy activation. However, the effect remained difficult to quantify and differences among strains were difficult to establish. Few studies have so far evaluated the capacity of probiotics or commensal bacteria to activate autophagy. Lin et al. have reported that four different bifidobacteria were able to trigger autophagy in the epithelial IEC-18 rat cell line [43]. However, they did not correlate this ability to functional properties. A bioactive lysate derived from a *Lactobacillus fermentum* strain was also shown in vitro to activate autophagy in the human hepatoma cell line HepG2 and correlated to an in vitro protective effect against drug-induced toxicity [44]. The activation of autophagy was also reported with *Bacillus amyloliquefaciens* SC06 in the murine macrophage RAW264.7 cell line and was shown to be involved in the antibacterial activity of the probiotic which promoted the elimination of an *E. coli* strain [45]. Conversely, the probiotic *L. rhamnosus* GG strain was shown to inhibit *Salmonella enterica* serovar infantis-induced autophagy, which, in turn suppressed intestinal epithelial cell death and limited *Salmonella* translocation in LGG-pre-treated pigs [46]. In the same way, supernatant from a culture of a *B. bifidum* strain inhibited LPS-induced autophagy in IEC-18 cells and alleviated LPS-induced intestinal epithelial cell injury [47]. All these data
could indicate that probiotic-induced autophagy reactions may be different according to the strains used, the host cells tested or the different pathophysiological contexts, albeit useful as a novel mechanism through which they are able to promote and maintain gut homeostasis.

Autophagy is emerging as one of the critical effector mechanisms of the gut immune homeostasis, influencing antigen presentation by DCs [22], the inhibition of IL-1B signaling [48], the regulation of inflammasome activation [16, 49] and Paneth cell function [17], among others [50]. We therefore evaluated the role of autophagy in the anti-inflammatory capacities of the selected probiotic strains. We showed that blocking the autophagy machinery using 3-MA or bafilomycin, respectively decreased or abandoned the release of the anti-inflammatory cytokine IL-10 on PBMCs, following stimulation with different lactobacilli and bifidobacteria. Conversely, the blockage of autophagy led to a strong increase of IL-1β secretion, notably with the strains that induced low or moderate basal levels of this pro-inflammatory cytokine (B. bifidum IPL A7.31, B. longum CMUL CXL 001, L. rhamnosus IPL A2.21, L. gasseri IPL A6.33 and L. reuteri 100-23). Also here the effect was more pronounced with bafilomycin. We observed a similar effect using BMDCs. Increased IL-1β production, following the blocking of autophagy or the loss of autophagy due to ATG16L1 deficiency in monocytes/macrophages, has been reported for the first time by Saitoh et al., suggesting that autophagy controls inflammasome activation. Even if 3-methyladenine (3-MA), a class-III PI3K inhibitor, is the first autophagy inhibitor identified and widely used to demonstrate the role of autophagy [51], some data reported that this compound can also have stimulatory effects when used as prolonged period of treatment under nutrient-rich conditions [52]. In the same way, bafilomycin A1, a V-ATPase inhibitor, may have indirect effects on any acidified compartment [14]. This suggests that we cannot rule out autophagy-independent effects in our conclusions.

The levels of the proinflammatory cytokines IL-1β and IL-18 were also significantly elevated in the sera of DSS-treated *atg16l1*-deficient mice in comparison to the levels in the wild-type counterparts [16]. The authors confirmed by 3-MA administration, that blocking autophagy increased the levels of IL-1β in serum and worsened the survival rate of mice treated with DSS, suggesting that autophagy protects mice from massive inflammation during colitis. This has been confirmed in human PBMCs, showing that the inhibition of autophagy increased IL-1β production after toll-like receptor-2 (TLR2) or TLR4 stimulation, albeit through an inflammasome-independent pathway [53]. Inflammasomes are cytosolic multiprotein complexes involved in the release of inflammatory cytokines after sensing invading pathogens. The activation of pattern recognition receptor (PRRs), such as Nod-like receptors (NLRs), triggers the assembly of inflammasome complexes, which lead to the recruitment of the Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD (ASC). The latter induces the activation of caspase-1 involved in the cleavage of the inactive precursors of IL-1β and IL-18. The subsequent maturation of these pro-inflammatory cytokines at the sites of infection or injury involves various physiological responses, including the recruitment of pro-inflammatory cells. Among the NLR inflammasome complexes, the NLRC3 inflammasome is the most widely characterized, as it is considered to be a crucial signaling pathway that controls the maturation of IL-1β and IL-18. A balanced regulation of the inflammasome activation and of induction of the IL-1 cytokine family is crucial to maintain immune homeostasis, influencing antigen presentation by DCs [22], the inhibition of IL-1B signaling [48], the regulation of inflammasome activation [16, 49] and Paneth cell function [17], among others [50]. We therefore evaluated the role of autophagy in the anti-inflammatory capacities of the selected probiotic strains. We showed that blocking the autophagy machinery using 3-MA or bafilomycin, respectively decreased or abandoned the release of the anti-inflammatory cytokine IL-10 on PBMCs, following stimulation with different lactobacilli and bifidobacteria. Conversely, the blockage of autophagy led to a strong increase of IL-1β secretion, notably with the strains that induced low or moderate basal levels of this pro-inflammatory cytokine (B. bifidum IPL A7.31, B. longum CMUL CXL 001, L. rhamnosus IPL A2.21, L. gasseri IPL A6.33 and L. reuteri 100-23). Also here the effect was more pronounced with bafilomycin. We observed a similar effect using BMDCs. Increased IL-1β production, following the blocking of autophagy or the loss of autophagy due to ATG16L1 deficiency in monocytes/macrophages, has been reported for the first time by Saitoh et al., suggesting that autophagy controls inflammasome activation. Even if 3-methyladenine (3-MA), a class-III PI3K inhibitor, is the first autophagy inhibitor identified and widely used to demonstrate the role of autophagy [51], some data reported that this compound can also have stimulatory effects when used as prolonged period of treatment under nutrient-rich conditions [52]. In the same way, bafilomycin A1, a V-ATPase inhibitor, may have indirect effects on any acidified compartment [14]. This suggests that we cannot rule out autophagy-independent effects in our conclusions.

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To better address the role of autophagy in the anti-inflammatory capacity of selected strains, we evaluated the impact of an in vivo blockage using 3-MA concomitantly with oral administration of two Lactobacillus strains in the murine model of TNBS-induced colitis. We selected one strain that we previously showed to be protective through an induction of immune-regulatory response (L. rhamnosus A2.21) together with the capacity to restore the epithelial barrier, and the strain L. acidophilus CMUL067, which exhibited protective effect essentially through an important strengthening of the epithelial barrier and the restoration of TJ expression [39]. As expected, strains were able to counteract the TNBS-induced colitis, with a significant reduction of macroscopic and histological scores of inflammation, of the plasmatic levels of IL-6 and of the colonic mRNA expression levels of the pro-inflammatory cytokines and chemokines measured. The protective effects of L. rhamnosus A2.21 were significantly inhibited after treatment with 3-MA. As reported using pharmacological stimulation of autophagy [42], we can conclude that L. rhamnosus A2.21 strain can reduce gut inflammation by a mechanism involving autophagy, although other mechanisms cannot be ruled out. Moreover, a huge increase of the plasmatic level of IL-1β was observed in the mice that received the 3-MA, indicating that a loss of control of the inflammasome occurred after blocking the autophagy machinery, as previously reported [16, 42]. Interestingly, the blockage of autophagy did not affect the protective capacity of L. acidophilus CMUL067. This suggests that autophagy is involved in the immuno-regulatory capacity of lactobacilli but not in the protective abilities linked to strengthening the gut barrier functions. Epithelial autophagy has been implicated in barrier enforcement during Salmonella infection. Indeed autophagy deficiency in intestinal epithelial cells (IECs) led to increased pathogenic bacterial dissemination and inflammation [57, 58]. Using tissue-specific atg16l1 deficient mice, Pott et al. demonstrated that autophagy within the intestinal epithelium but not in myeloid cells, is involved in the maintenance of barrier integrity and limits inflammation in a model of chronic colitis [59]. Epithelial autophagy might also be a response mechanism to mucosal barrier dysfunction in severe acute pancreatitis, by preventing and reducing the oxidative stress. A cell-type-specific function of autophagy has been reported in the context of mucosal homeostasis. Impairment of autophagic function in IECs has been related mainly to Paneth cell dysfunction [60]. In myeloid cells, autophagy is implicated in regulation of proinflammatory cytokine response, particularly in secretion of inflammasome-dependent cytokines and ROS levels [16, 61]. Recent studies also revealed that autophagy is required for the survival and function of regulatory T-cells in the gut, important to maintain intestinal homeostasis [28]. Multiple cell types, notably epithelial versus hematopoietic cells, interact with commensal bacteria and can mount different and complementary functions in the gut. We previously observed that selected lactobacilli partially activated BMDCs, inducing regulatory DCs able to confer protection towards TNBS-induced colitis after adoptive transfer, in a NOD2-dependent manner [32, 62]. The protective effect was linked to the development of CD4+Foxp3+ regulatory T cells. Since DCs, the most potent antigen-presenting cells, are critical in initiating and maintaining immune homeostasis and tolerance, we investigated how autophagy can impact their function. As previously observed for a Lactobacillus salivarius strain, the adoptive transfer of BMDCs pulsed in vitro with L. rhamnosus A2.21 was able to confer protection in TNBS-treated mice, while untreated DCs had no effect. When DCs were stimulated by the bacteria in the presence of the autophagy inhibitor 3-MA, they were no longer able to rescue mice from colitis. This was also confirmed using L. rhamnosus-pulsed atg16l1-deficient BMDCs, which lost their protective capacity in comparison to L. rhamnosus-pulsed DCs derived from WT or control mice. This indicated that autophagy is involved in the capacity of the selected strain to induce regulatory DCs and that this cellular process is important for DCs to exhibit their function in the gut.

Autophagy has been shown to be involved in DC functions at several levels: antigen presentation, cytokine production, DC migration and maturation and T-cell activation. Notably, autophagy has been associated with a positive role in the tolerogenic maturation of DCs [27]. Specific disruption of autophagy in CD11c+ DCs exacerbated DSS-induced murine colitis with increased induction of the pro-inflammatory cytokines IL-1β and TNF-α. This
Atg16l1 deficiency led to increase in ROS production and was proposed as a novel protective pathway during gut inflammation [26]. Using DC-IEC co-culture on transwell inserts, it has also been demonstrated that the blockage of autophagy through the use of siRNA led to abnormal DC-IEC interactions [63].

Conclusion

Considering the complex cross talk between the host and the gut microbiota, it is now well recognized that autophagy can be involved in gut homeostasis maintenance at different levels. Our study has revealed for the first time that this cellular machinery is involved in the anti-inflammatory capacities of selected potential probiotic strains and particularly in their capacities to induce immune-regulatory responses, notably regulatory DCs. Even if it remains important to dig deeper into the mechanisms involved, we can conclude that autophagy should be added as a novel criteria for probiotic selection in the management of IBD.

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Author contributions

MZ conceived the design and realization of the experimental work, analysis and interpretation of the data, discussion and writing of the manuscript. CG coordinated the work, participated to the animal experimental procedures, analysis of results, discussion and writing of the manuscript. JA initiated the work and participated to experimental experiments. VP, DB and JD participated to animal and cellular experiments. PR and HG provided the atg16l1-deficient mice and participated to the discussion and writing of the manuscript. BP, IAK, FD helped in the discussion and writing of the manuscript. All the authors contributed to the critical review of the manuscript and approved its final version.

Disclosure Statement

The authors declare that they have no conflicts of interests.

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