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ORIGINAL ARTICLE

Combined probiotic bacteria promotes intestinal epithelial barrier function in interleukin-10-gene-deficient mice

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Abstract

AIM: To investigate the protective effects of combinations of probiotic (Bifico) on interleukin (*IL*)-*10*-gene-deficient (*IL-10* KO) mice and Caco-2 cell monolayers.

METHODS: IL-10 KO mice were used to assess the benefits of Bifico *in vivo*. IL-10 KO and control mice received approximately 1.5×10^8 cfu/d of Bifico for 4 wk. Colons were then removed and analyzed for epithelial barrier function by Ussing Chamber, while an ELISA was used to evaluate proinflammatory cytokines. The colon epithelial cell line, Caco-2, was used to test the benefit of Bifico *in vitro*. Enteroinvasive *Escherichia coli* (EIEC) and the probiotic mixture Bifico, or single probiotic strains, were applied to cultured Caco-2 monolayers. Barrier function was determined by measuring transepithelial electrical resistance and tight junction protein expression.

RESULTS: Treatment of IL-10 KO mice with Bifico partially restored body weight, colon length, and epithelial barrier integrity to wild-type levels. In addition, IL-10 KO mice receiving Bifico treatment had reduced mucosal secretion of tumor necrosis factor-α and interferon-γ, and attenuated colonic disease. Moreover, treatment of Caco-2 monolayers with Bifico or single-strain probiotics *in vitro* inhibited EIEC invasion and reduced the secretion of proinflammatory cytokines.

CONCLUSION: Bifico reduced colon inflammation in IL-10 KO mice, and promoted and improved epithelial-barrier function, enhanced resistance to EIEC invasion, and decreased proinflammatory cytokine secretion.

Key words: Probiotic bacteria; Intestinal barrier function; Tight junction proteins; Interleukin-10 gene-deficient mice; Caco-2 monolayers

Core tip: We investigated the protective effects of combinations of probiotic bacteria (Bifico) on interleukin (IL)-10 gene-deficient (IL-10 KO) mice and Caco-2 cell monolayers. Treatment of IL-10 KO mice with Bifico partially restored body weight, colon length, and epithelial barrier integrity to wild-type levels. Treatment of Caco-2 monolayers with Bifico or single-strain probiotics inhibited enteroinvasive *Escherichia coli* (EIEC) invasion and reduced secretion of proinflammatory cytokines. Oral administration of Bifico reduced colon inflamma-

tion, and directly promoted epithelial barrier function. In addition, Bifico improved epithelial barrier function, and enhanced resistance to EIEC invasion *in vitro*.

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INTRODUCTION

The human gut is colonized with a wide variety of microorganisms, including pathogenic, probiotic and commensal bacteria. *Bifidobacterium, Lactobacillus* and *Enterococcus faecalis* are probiotics with beneficial effects on maintenance therapy of human intestinal diseases[1-3]. For example, oral treatment with specific probiotic bacteria can ameliorate inflammatory bowel disease (IBD). In addition, multiple studies have demonstrated that colonization strategies using defined commensals or exogenous specific probiotic treatment may prevent host intestinal inflammation and ameliorate intestinal epithelial barrier function[3-9].

The intestinal barrier prevents microbial contamination of interstitial tissues. Tight junctions play an important role in modulating intestinal epithelial paracellular permeability[10] and promoting defenses against harmful molecules and microorganisms. Tight junctions are common targets of enteric pathogens, and their disruption occurs with IBD and/or diarrhea[11]. For example, many virulence genes of bacteria encode toxins and other proteins that either directly disassemble tight junction proteins[12,13] or modulate intracellular pathways that lead to tight junction redistribution[14,15]. Enteroinvasive *Escherichia coli* (EIEC)[12,13], *Salmonella typhimurium*[16], *Shigella flexneri*[17,18], and *Campylobacter jejuni*[14,15] all infect host cells by targeting the paracellular pathway.

Past studies have demonstrated that IBD patients have reduced bifidobacteria and lactobacilli in their gut microbiota[19,20] suggesting these patients may benefit from probiotic treatment. Indeed, recent clinical trials have confirmed the therapeutic effects of probiotics in virus-, bacterium-induced intestinal infections and antibiotic-induced diarrhea[21-23]. Among the most distinctive benefits of probiotic bacteria are modulation of host defense responses, and protection against infectious diseases[24,25]. However, the molecular mechanisms underlying these effects have not fully been elucidated.

The probiotic compound, Bifico (Bifico Pharmaceuticals, Sine, Shanghai, China), contains about 1.0×10^9 cfu/g of viable lyophilized bifidobacteria (*Bifidobacterium longum*), 1.0×10^9 cfu/g lactobacilli (*Lactobacillus acidophilus*), and 1.0×10^9 cfu/g *Ent. faecalis*. This probiotic combination has been effective in the maintenance therapy of diarrhea induced by intestinal flora disturbance or enteritis. However, the use of Bifico as a primary therapy for IBD has not yet been investigated. To address this issue, we treated interleukin (IL)-10-gene-deficient (IL-10 KO) with Bifico and monitored the presence of IBD, which spontaneously develops in these mice. In addition, Caco-2 cells were cultured *in vitro* with EIEC with or without Bifico pretreatment to monitor EIEC invasion. Bifico had a direct effect on epithelial barrier function *in vivo* by reducing mucosal secretion of tumor necrosis factor (TNF)-α and interferon (IFN)-γ, and altered the expression and distribution of tight junction proteins. Bifico exposure *in vitro* reduced bacterial invasion. Moreover, the effects of combined probiotics were more pronounced than single-strain probiotics.

MATERIALS AND METHODS

Animals

Homozygous IL-10 KO mice, generated on a 129 Sv/Ev background, and normal 129 Sv/Ev controls (Jackson Laboratory, Bar Harbor, ME, United States) were housed under specific-pathogen-free conditions in Shanghai Jiao Tong University Medical School. Mice were fed a standard sterile diet and filtered water *ad libitum* under a 12-h light-dark cycle. Animal studies were approved by the Ethical Committee of the Affiliated Sixth People's Hospital of Shanghai Jiao Tong University.

In vivo Bifico therapy

Ten-week-old female IL-10 KO mice (n = 12) and wild-type (WT) controls (n = 12) were randomized and divided into two groups each. Mice then either received a daily oral gavage of Bifico dissolved in 0.5 mL physiological saline at 3.0×10^8 cfu/mL or saline alone for 4 wk.

Ussing Chamber assay

Mice were sacrificed following Bifico therapy, and a segment of the colon was removed for mucosa isolation from the muscular layer. Mucosal cells were mounted in Lucite Chambers, exposing mucosal and submucosal surfaces to 10 mL oxygenated Krebs Buffer (EasyMount-CSYS-8 Using Chamber Systems; San Diego, CA, United States). The buffers were maintained at 37 ℃ by a heated water jacket and circulated in CO_2/O_2[26]. The nonabsorbable tracer molecule inulin-FITC (2000-5000 kDa, Sigma-Aldrich, St Louis, MO, United States) (1.0 mg/mL) was then added to the mucosal side of the Lucite Chambers. At 0, 30, 60, 90 and 120 min following addition of inulin-FITC, 100 μL buffer from the submucosal side of the Lucite Chambers was collected and analyzed for fluorescence in black-walled 96-well plates (Costar, Corning, NY, United States) using a Spectral Scanning Multimode Reader (Thermo Scientific Varioskan Flash, Vantaa, Finland) at an excitation wavelength of 485 nm and emission at 530 nm[27,28]. Standard curves were obtained by diluting inulin-FITC in Krebs Buffer. The barrier function of the intestinal epithelium was also determined by measuring

transepithelial electrical resistance (TER) (multi-channel voltage current clamp, VCC MC8, San Diego, CA, United States).

Histological injury grading

Mice were sacrificed after 4 wk Bifico treatment. Colons were harvested and fixed in 10% phosphate-buffered formalin. The samples were paraffin-embedded, sectioned at 5 μm, and stained with hematoxylin and eosin (HE) for microscopic examination and imaging (Nikon Eclipse 80i, Tokyo, Japan). The slides were reviewed in a blinded fashion by two pathologists and were assigned a histological score for intestinal inflammation based on previously described criteria[29,30]. No inflammation was scored as 0; modest numbers of infiltrating cells in the lamina propria as 1; infiltration of mononuclear cells leading to separation of crypts and mild mucosal hyperplasia as 2; massive infiltration with inflammatory cells accompanied by disrupted mucosal architecture, loss of goblet cells, and marked mucosal hyperplasia as 3; these issues plus crypt abscesses or ulceration as 4; with a total score from 0 to 15.

Transmission electron microscopy

To observe the ultrastructural changes of tight junctions, colonic segments were prepared for transmission electron microscopy (JEM1230 Electro-microscope; JEOL, Tokyo, Japan) as previously described[8].

ELISA

The serum levels of TNF-α and IFN-γ were measured using ELISA kits (BD Pharmingen, Oxford, United Kingdom) as previously described[8]. The levels of the cytokines TNF-α, macrophage inflammatory protein (MIP)-1α, IL-6 and IL-8 in the supernatant of cultured Caco-2 monolayers were measured using Sandwich ELISA kits (R&D, Minneapolis, MN, United States) according to manufacturer's instructions.

Caco-2 monolayers

Caco-2 cells (Shanghai Institute of Cell Biology, Chinese Academy of Science) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, United States) supplemented with 100 mL/L heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured in 25-cm^2 flat-bottom culture flasks (Corning, Corning, NY, United States) and seeded onto Transwell semipermeable filters (filter grown; 1.12 cm^2 polyester membranes, 3.0 μm pore size, 12 well) in Transwell units (Costar; Corning). The barrier function of tight junctions in Caco-2 monolayers was determined by measuring TER.

Bacterial cultivation

B. longum, *L. acidophilus*, and *Ent. faecalis* were obtained from Shanghai Sine Pharmaceutical Co. Ltd. EIEC (O124: NM, ATCC 43893) was obtained from the Shanghai Municipal Center for Disease Control and Prevention. *B. longum* was cultured in tryptone polypeptone yeast extract broth agar (Shanghai Sine Pharmaceutical Co. Ltd) at 37 ℃. *Ent. faecalis* was cultured in Slanetz and Bartley agar (Shanghai Sine Pharmaceutical Co. Ltd.) at 37 ℃. *L. acidophilus* was cultured in MRS agar (Merck, Darmstadt, Germany) at 37 ℃. EIEC was cultured in LB agar (Oxoid, Hampshire, United Kingdom) at 37 ℃. Bacteria were added to DMEM and then subjected to photoelectric colorimeter to measure consistency.

Infection of Caco-2 monolayers with EIEC

Caco-2 cells were washed three times in Hank's Solution to remove antibiotics. The inoculation ratio of EIEC to Caco-2 cells was approximately 100:1. Prior to EIEC infection, four groups of Caco-2 monolayers were incubated with *B. longum* (B), *Ent. faecalis* (F), *L. acidophilus* (L) or the triple bacteria (BFL) for 30 min. The ratio of probiotics to EIEC was 10:1. EIEC was allowed to infect Caco-2 monolayers for 24 h. TER of Caco-2 monolayers was measured with a voltmeter (Millicell-ERS; Millipore, CA, United States) for 6 h. Untreated Caco-2 monolayers served as a control (C). Caco-2 cells infected with EIEC alone served as the EIEC (E) group. Incubation medium was collected and processed for cytokine analysis using Sandwich ELISA kits (R&D).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), western blotting and immunofluorescence

Tissue total RNA (SLNco, Shanghai, China) was extracted and analyzed by qRT-PCR (Funglyn, Ontario, Canada) as previously described[8,13]. Rabbit polyclonal antibodies against zonula occludentes 1 (ZO-1, Invitrogen), claudin-1 (Invitrogen) and occludin (Invitrogen) were used in western blotting and immunofluorescence assays according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using the GraphPad Prism 5 software (San Diego, CA, United States) and expressed as mean ± SEM. Differences in parametric data were evaluated by Student's two-tailed unpaired *t* test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Bifico reduced clinical disease and prevented colonic epithelial permeability in IL-10 KO mice

Bifico therapy in IL-10 KO mice was used to assess the effects of probiotics in reducing IBD. As seen in the representative photomicrographs of Figure 1, the morbidity in IL-10 KO mice was greater than in IL-10 KO mice treated with Bifico (Figure 1A). During the 4-wk observation, IL-10 KO mice displayed a significant reduction in body weight compared to WT mice. However, Bifico treatment restored the body weight in IL-10 KO mice (Figure 1B). In addition, the presence of diarrhea was greater in IL-10 KO mice than in mice receiving Bifico

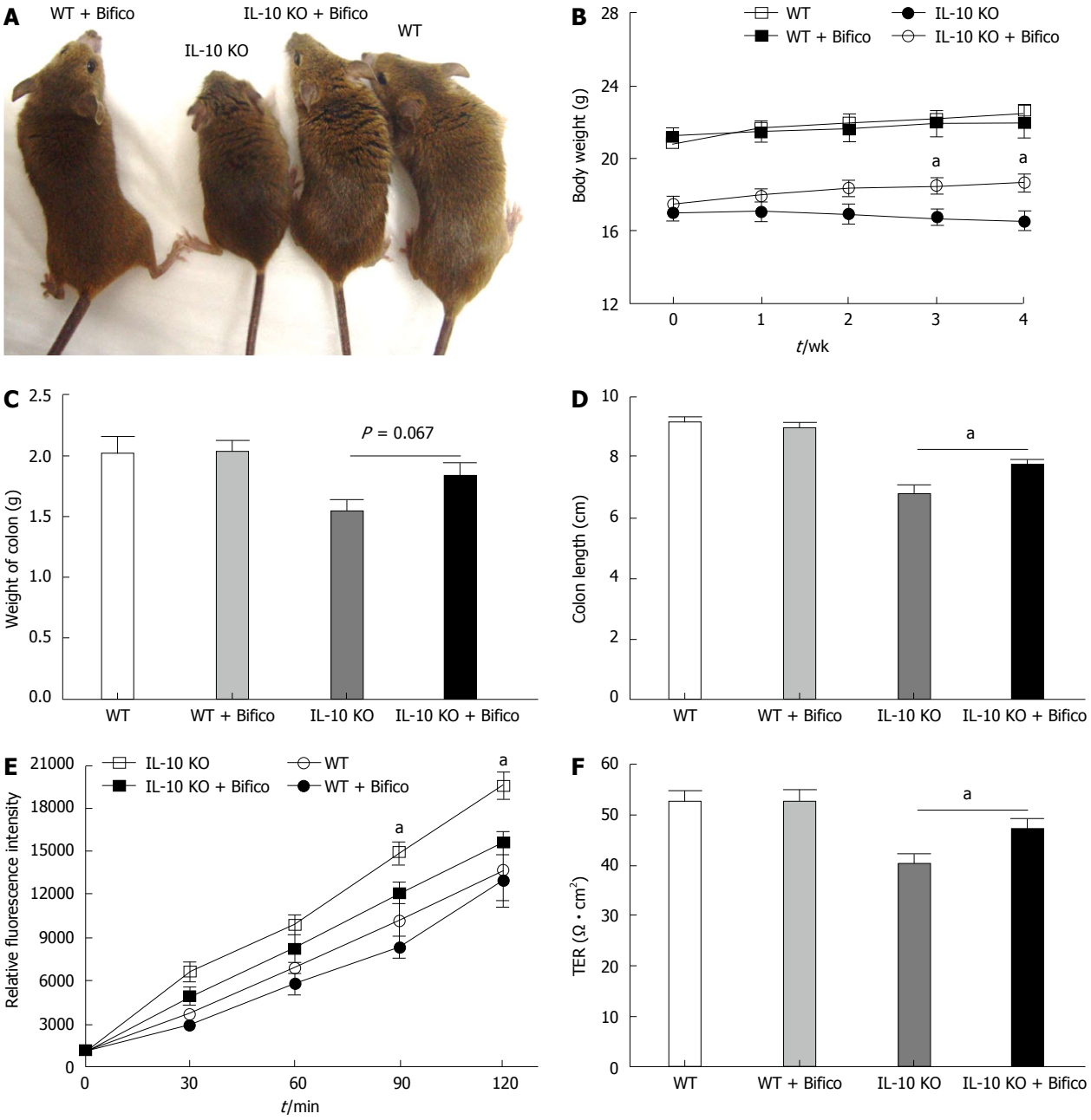

Figure 1 Bifico reduced clinical disease activity and prevented colonic epithelial permeability in interleukin-10 gene-deficient mice. A: Representative photographs from the indicated mice for 4 wk following Bifico treatment; B: Changes in body weight. The IL-10 KO group was significantly lighter than the IL-10 KO + Bifico group (mean ± SEM, n = 6 per time point per group, $^aP < 0.05$, Student's t test); C: Colon weight were measured at 28 d following Bifico treatment; D: Colon length was measured at 28 d following Bifico treatment. IL-10 KO mice had shorter colons than IL-10 KO mice treated with Bifico. No difference in colon weight was observed (mean ± SEM, n = 6 per group, $^aP < 0.05$, Student's t test); E: Colonic paracellular permeability measured by cumulative permeability of nonabsorbable tracer molecule inulin-FITC; F: Colonic paracellular permeability measured by TER. IL-10 KO mice presented higher permeability than IL-10 KO mice treated with Bifico (mean ± SEM, n = 5 per time point per group, $^aP < 0.05$, Student's t test).

treatment. The colonic weight did not differ significantly between Bifico-treated or untreated IL-10 KO mice (Figure 1C); however, the colons of IL-10 KO mice were significantly shorter than those of IL-10 KO receiving Bifico treatment (Figure 1D).

To assess the effects of Bifico on colon function, we measured colonic permeability by Ussing Chamber. IL-10 KO mice exhibited a significant increase in the cumulative permeation of inulin-FITC through the colonic mucosa compared with WT and Bifico-treated WT mice (Figure 1E). In accordance with increased inulin-FITC permeability, a significant decrease of TER was observed in IL-10 KO mice (Figure 1F). However, Bifico treatment restored colon function in IL-10 KO mice (Figure 1E and F).

Bifico therapy ameliorated inflammation and reduced production of proinflammatory cytokines

To evaluate further the effects of Bifico *in vivo*, mice were sacrificed following Bifico therapy and mucosal cells

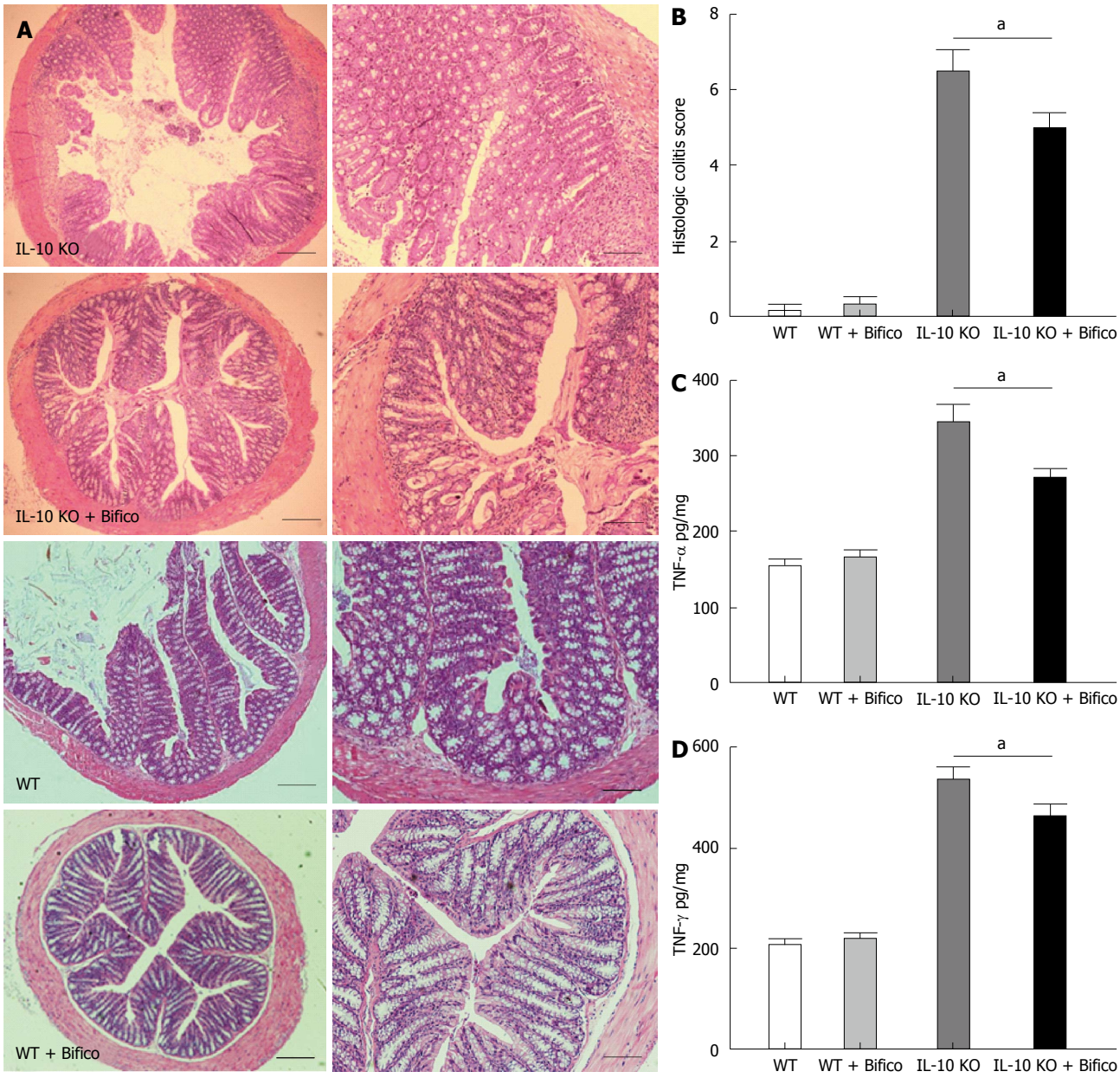

Figure 2 Bifico attenuated inflammation and production of proinflammatory cytokines. A: Representative photomicrographs showing colonic inflammation and damage (HE stain, × 40 magnification, higher magnification photomicrographs on the right × 100); B: Histological colitis scores (mean ± SEM, $n = 6$ per group, $^aP < 0.05$, Student's t test); C and D: TNF-α and IFN-γ were measured by ELISA in colon after 4 wk Bifico treatment (mean ± SEM, $n = 6$ per group, $^aP < 0.05$, Student's t test).

were evaluated by histology. HE staining revealed a large amount of mucosal damage and inflammatory cell infiltration in the lamina propria of IL-10 KO mice (Figure 2A). In addition, all IL-10 KO mice developed colitis, and the inflammatory score was significantly greater in IL-10 KO than WT mice. However, IL-10 KO mice receiving Bifico treatment had reduced inflammatory scores with only mild cell infiltration and mucosal damage (Figure 2B).

We next evaluated the secretion of proinflammatory cytokines from colonic tissue *ex vivo*. Expression of TNF-α and IFN-γ in the colonic mucosa was significantly increased in IL-10 KO mice (Figure 2C and D). However, 4 wk Bifico treatment significantly reduced the levels of TNF-α and IFN-γ (Figure 2C and D). These data suggest that Bifico treatment reduces the induction of IBD in IL-10 KO mice.

Bifico therapy altered apical junction protein expression and distribution

To identify whether there were changes in apical junction protein expression and distribution following Bifico treatment, immunofluorescence, western blotting and real-time qRT-PCR assays of ZO-1, claudin-1 and occludin were performed on the colonic tissue. There were significantly decreased in the expression of protein and mRNA of ZO-1, claudin-1 and occludin. As expected, the network of ZO-1, claudin-1 and occludin was predominantly intact and localized along the apical cellular border in WT mice. In IL-10 KO mice, the expression of ZO-1, claudin-1

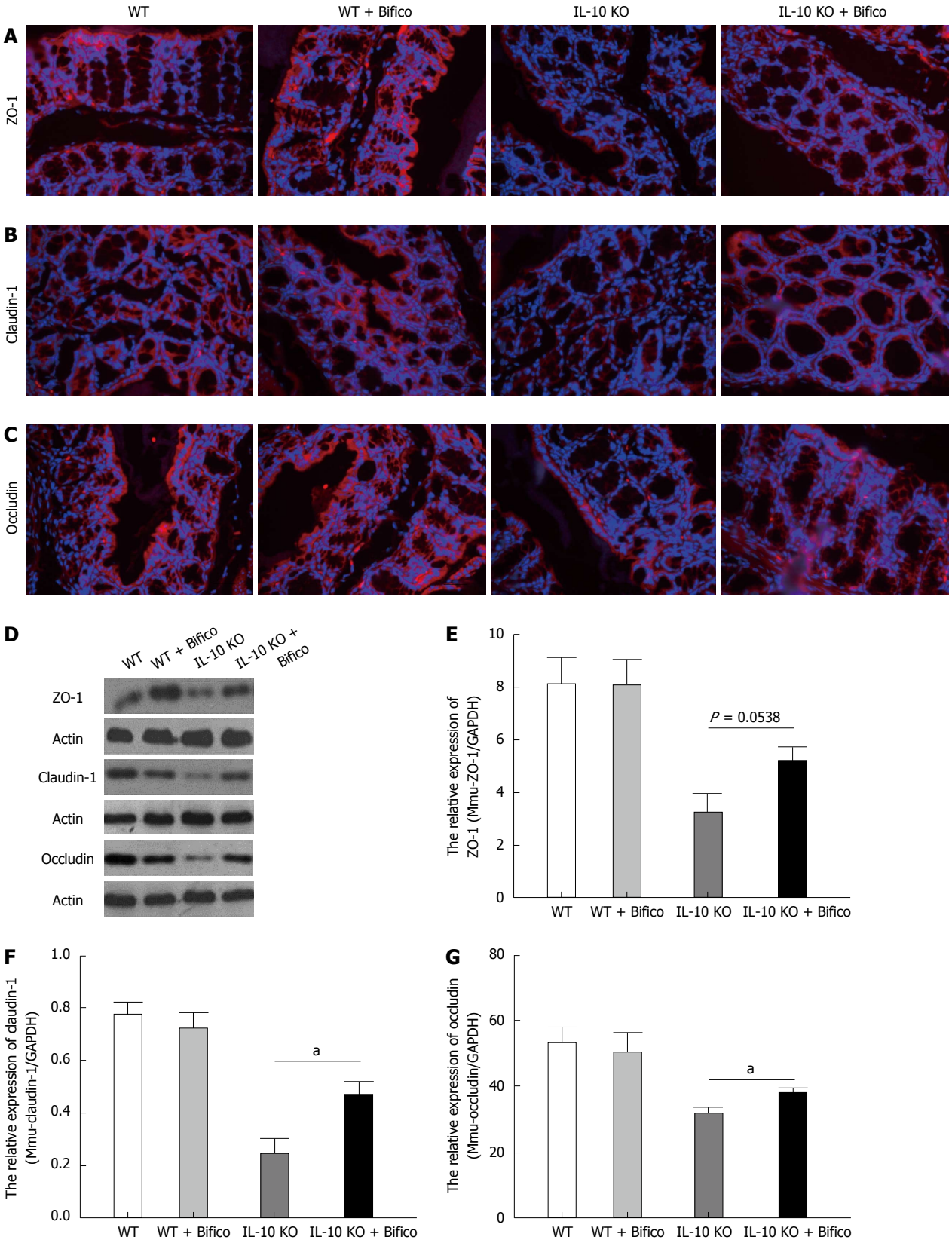

Figure 3 Bifico increased expression of tight junction proteins in interleukin-10 gene-deficient mice. A: Representative immunofluorescence photomicrographs for tight junction proteins zonula occludentes (ZO)-1, in the colonic epithelium (n = 3 for each group); B: Representative immunofluorescence photomicrographs for tight junction proteins claudin-1 (n = 3 for each group); C: Representative immunofluorescence photomicrographs for tight junction proteins occludin (n = 3 for each group).The tight junction proteins were stained red (× 400 magnification); D: Western blotting of tight junction proteins in the colonic tissues; E: mRNA expression of ZO-1 in the colon (mean ± SEM, n = 4-6 per group, $^aP < 0.05$, Student's t test); F: mRNA expression of claudin-1 in the colon (mean ± SEM, n = 4-6 per group, $^aP < 0.05$, Student's t test); G: mRNA expression of occludin in the colon (mean ± SEM, n = 4-6 per group, $^aP < 0.05$, Student's t test).

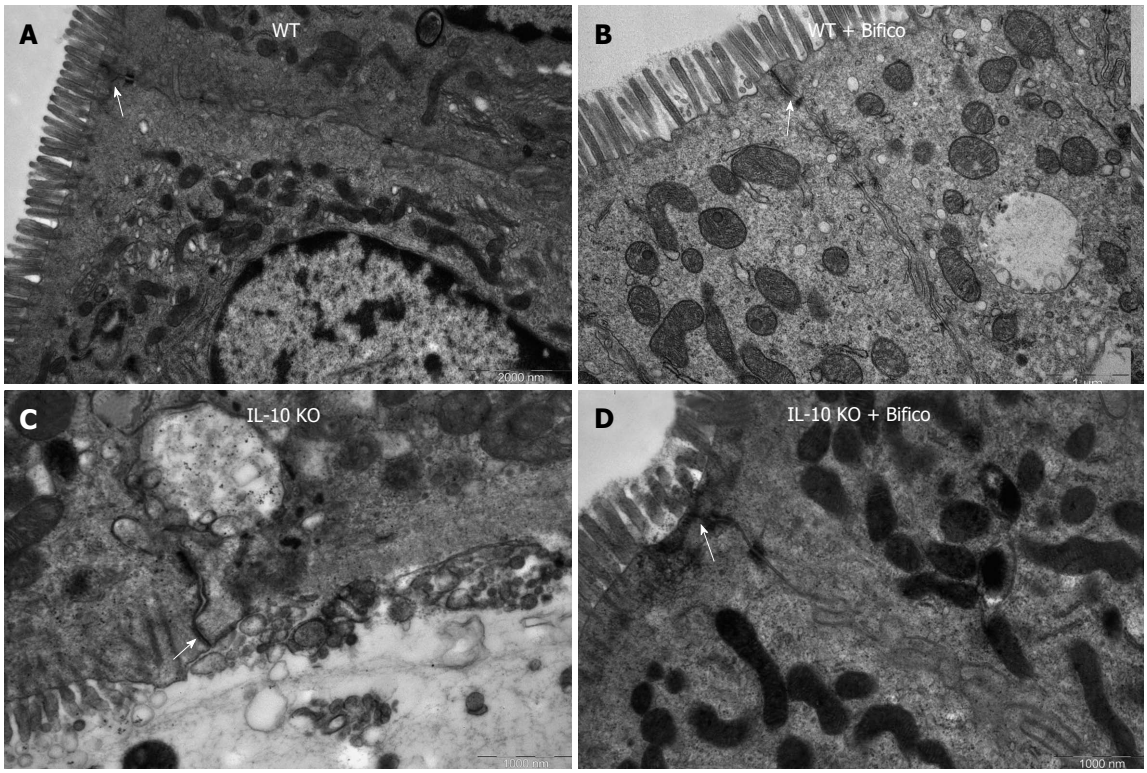

Figure 4 Bifico ameliorated the ultrastructure of colonic epithelium in interleukin-10 gene-deficient mice. A: Intercellular tight junctions (indicated by the white arrows) and normal ultrastructure in colonic epithelium of WT (scale bar = 2000 nm); B: Intercellular tight junctions (indicated by the white arrows) and normal ultrastructure in colonic epithelium of Bifico treated WT mice (scale bar = 1000 nm); C: Tight junctions (indicated by the white arrows) and microvilli, increased intercellular gap, and abnormal ultrastructure of epithelial cells in the colonic epithelium of IL-10 KO mice (scale bar = 1000 nm); D: Partly recovered intercellular tight junctions (indicated by the white arrows) and microvilli in colonic epithelium of Bifico treated IL-10 KO mice (scale bar = 1000 nm).

and occludin at the apical cellular border was decreased, discontinuous, and redistributed. Bifico treatment ameliorated these changes in IL-10 KO mice (Figure 3A-C).

Western blotting and RT-PCR showed that the protein and mRNA levels of ZO-1, claudin-1 and occludin were reduced in IL-10 KO mice when compared with WT mice (Figure 3D). Four weeks of Bifico therapy restored the protein and mRNA levels of ZO-1, claudin-1 and occludin in IL-10 KO mice (Figure 3E-G). These data suggest that Bifico treatment promotes proper tight junction protein expression and distribution.

Bifico therapy normalized ultrastructure in colonic epithelium of IL-10 KO mice

Colonic epithelial paracellular permeability is controlled mainly by tight junction proteins. Given that Bifico treatment restored tight junction distribution, we examined the ultrastructural changes of intercellular tight junction proteins by transmission electron microscopy. Compared with WT controls (Figure 4A and B), the colonic epithelium of IL-10 KO mice displayed significant ultrastructural changes in tight junction proteins and microvilli, along with marked increases in intercellular gaps and intracellular vacuolization (Figure 4C). However, Bifico therapy increased intact intercellular tight junctions and microvilli and decreased intercellular gaps and intracellular vacuolization (Figure 4D).

Probiotic treatment increased expression of tight junction proteins in EIEC-treated Caco-2 monolayers

Caco-2 monolayers were incubated with EIEC alone, or were pretreated with Bifico or single-strain probiotics for 24 h. Real-time qRT-PCR was then performed to analyze the expression of mRNA of ZO-1, claudin-1 and occludin in treated and untreated Caco-2 monolayers. Expression of ZO-1, claudin-1 and occludin mRNA was significantly decreased after treatment with EIEC in comparison to untreated controls. The decrease in tight junction proteins was inhibited by pretreatment with probiotics, with Bifico being the most effective (Figure 5A-C).

We next used western blotting to determine the relative proteins levels of ZO-1, claudin-1 and occludin in control and EIEC-treated Caco-2 monolayers incubated with or without various probiotics. Levels of tight junction proteins were markedly decreased after treatment with EIEC compared to untreated controls (Figure 5D-G). However, preincubation of Caco-2 cells with Bifico or single-strain probiotics significantly inhibited the reduction of tight junction proteins after EIEC infection, with Bifico being the most effective (Figure 5D-G). These data suggest that Bifico therapy can prevent EIEC

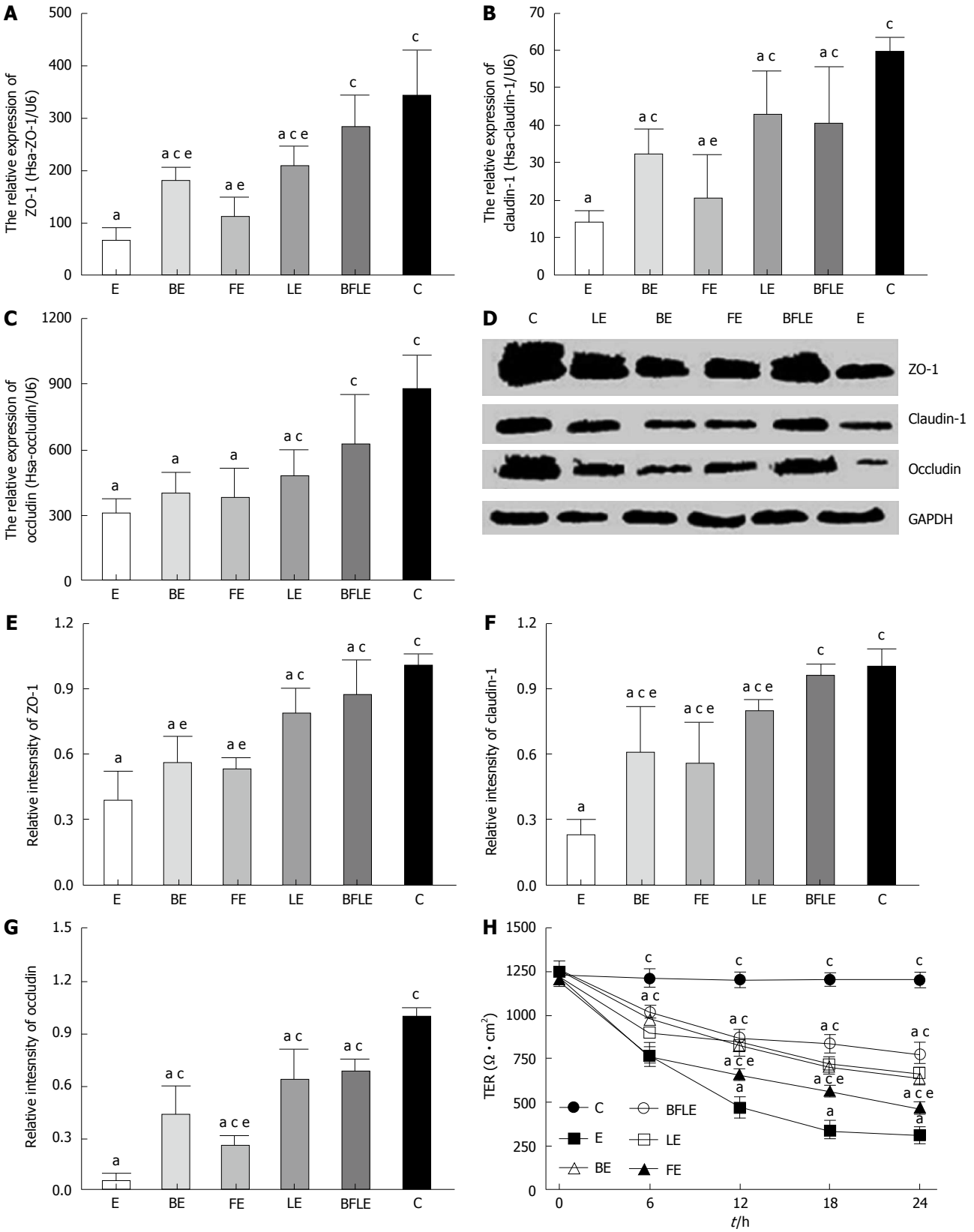

Figure 5 Bifico rescued expression of tight junction-associated proteins in following Enteroinvasive *Escherichia coli* infection. A: mRNA levels of tight junction proteins zonula occludentes (ZO)-1 were significantly decreased in Caco-2 monolayers after treatment with Enteroinvasive *Escherichia coli* (EIEC) in comparison to control untreated cells; B: mRNA levels of tight junction proteins claudin-1 were significantly decreased in Caco-2 monolayers; C: mRNA levels of tight junction proteins occludin were significantly decreased in Caco-2 monolayers. This decrease was reversed by pretreatment with Bifico or single strain probiotics; D: Representative experiment showing western blot (WB) analysis of ZO-1, claudin-1 and occludin expression; E: Expression of ZO-1 was quantified by densitometry for three independent experiments; F: Expression of cllaudin-1 was quantified by densitometry for three independent experiments; G: Expression of occludin was quantified by densitometry for three independent experiments; H: Bifico or single-strain probiotics increased the EIEC-treated TER of Caco-2 monolayers (means ± SEM, n = 3 per group in WB, n = 5 per group in qRT-PCR, n = 5 per time point per group in TER. $^{a}P < 0.05$ compared with control group, $^{c}P < 0.05$ compared with EIEC group, $^{e}P < 0.05$ compared with BFLE group, Student's t test). B. longum (B), E. faecalis (F), L. acidophilus (L), triple bacteria (BFL), control group (C), EIEC group (E). Preincubated with probiotics or/and commensal bacteria prior to EIEC infection created four groups (BE, FE, LE and BFLE).

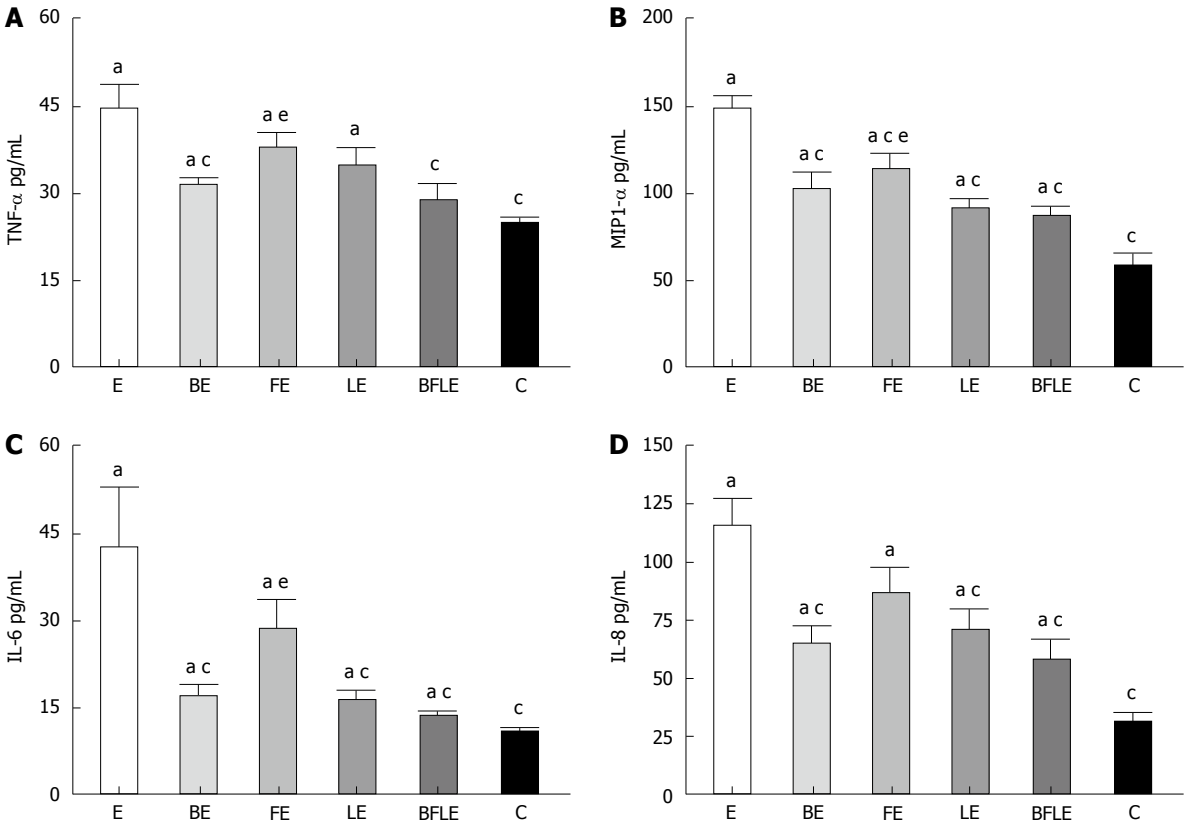

Figure 6 Bifico inhibited Enteroinvasive *Escherichia coli*-induced proinflammatory cytokines secretion. A: The level of TNF-α was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to Enteroinvasive *Escherichia coli* (EIEC) infection; B: The level of macrophage inflammatory protein (MIP)-1α was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to EIEC infection; C: The level of IL-6 was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to EIEC infection; D: The level of IL-8 was significantly inhibited by Bifico or single-strain probiotic pre-treatment of Caco-2 monolayers prior to EIEC infection (means ± SEM, n = 6 per group, $^{a}P < 0.05$ compared with control group, $^{c}P < 0.05$ compared with EIEC group, $^{e}P < 0.05$ compared with BFLE group, Student's t test).

infection by promoting tight junction protein expression.

Probiotic treatment increased TER in EIEC-treated Caco-2 monolayers

To investigate the effect of probiotics on EIEC-treated Caco-2 monolayers, we measured the TER of untreated and treated Caco-2 monolayers. Caco-2 monolayers were incubated with EIEC alone, or were pretreated with Bifico or single-strain probiotics for 30 min and then incubated with EIEC for 24 h. TER was measured for 6 h. Infection with EIEC resulted in an about 80% decrease in TER when compared with the untreated group. However, preincubation of Caco-2 monolayers with Bifico or single-strain probiotics attenuated TER reduction, with Bifico treatment being the most effective (Figure 5H).

Probiotics altered cytokine response in EIEC-treated Caco-2 monolayers

To address further the effects of probiotics on EIEC invasion, we measured the cytokine response in EIEC-treated Caco-2 monolayers. Caco-2 monolayers were incubated as described above, and the cytokines in the cell culture supernatant were evaluated by ELISA. The proinflammatory cytokine release from EIEC-treated Caco-2 monolayers was significantly inhibited by pretreatment with Bifico or single-strain probiotics (Figure 6). In addition, Bifico treatment was the most effective.

DISCUSSION

In the present study, we demonstrated that treating IL-10 KO mice with the probiotic mixture, Bifico, partly recovered colonic barrier integrity, reduced mucosal secretion of proinflammatory cytokines, and attenuated histopathological changes. Furthermore, *in vitro* studies revealed that epithelial barrier function and resistance to EIEC invasion was enhanced following exposure to Bifico.

The intestinal luminal microflora and their products are important initiating and modulating factors in the pathogenesis of IBD and some diarrhea diseases in humans and animals[31-34]. IL-10 KO mice spontaneously develop IBD, and this is associated with altered colonic microflora colonization[35]. These data suggest that probiotic therapy could reduce IBD in theses mice. Indeed, inoculation of *Lactobacillus* sp. reduced IBD in IL-10 KO mice. In the present study, we demonstrated that IBD was reduced in IL-10 KO mice by the probiotic mixture Bifico, and this reduction was greater than with the use of single-strain probiotics. These data are consistent with other *in vivo* experiments using VSL#3 (VSL Pharma-

ceuticals, Gaithersburg, MD, contains 9×10^{10} cfu/g of viable, lyophilized bifidobacteria (*B. longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*), 8×10^{10} lactobacilli (*L. acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *L. bulgaricus*, and *Lactobacillus plantarum*), and 20×10^{10} *Streptococcus salivarius* subsp. Thermophilus) compound strains, which are more effective than the use of a single *Lactobacillus* in improving colitis and normalizing epithelial function[3]. These data suggest that combinations of adherent probiotic strains can influence the adhesion and activity of other strains in the human intestinal tract[36]. Our study is believed to be the first to show that Bifico therapy is effective in ameliorating epithelial damage and restoring epithelial function in both *in vivo* and *in vitro* models.

In IL-10 KO mice, colonic inflammation is related to high levels of mucosal TNF-α and IFN-γ[32]. Recently, multiple studies have characterized the ability of various strains of probiotics to alter the activity and cytokine expression in the colons of IL-10 KO mice[3,8,37,38]. TNF-α and IFN-γ produced by colonic mucosa are both normalized when IL-10 KO mice are raised under germ-free conditions[32], suggesting that colitis in IL-10 KO mice occurs as a consequence of a Th1-predominant intestinal inflammation in the presence of the gut flora[3,8,37,38]. In present study, treating IL-10 KO mice with the probiotic mixture Bifico attenuated TNF-α and IFN-γ secretion. In addition, pretreatment of Caco-2 cells with Bifico significantly inhibited the secretion of TNF-α, MIP-1α, IL-6 and IL-8 following EIEC infection. These results indicate that the gut is able to discriminate and define selective responses to different bacteria.

To reduce inflammation, the normal intestinal barrier is impermeable to bacteria, bacterial products, and dietary antigens that are present within the lumen. Thus, probiotic bacteria may exert protection through enhancing intestinal barrier function, including decreasing intestinal permeability and maintaining normal expression and distribution of tight junction proteins[6,8]. IL-10 KO mice demonstrated increased colonic permeability that is absent in mice raised under germ-free conditions[32]. Bifico treatment, however, reduced epithelial permeability in IL-10 KO mice, and increased expression of tight junction proteins in both colonic epithelia *in vivo* and in EIEC-treated Caco-2 monolayers. Moreover, the use of the Bifico therapy was more effective than the use of a single-strain probiotics in preventing disruption and normalizing function of Caco-2 monolayers following EIEC infection.

Although Bifico therapy was the most effective, single-strain probiotics were also effective in restoring Caco-2 cells function following EIEC infection. These data suggest that epithelial cells may respond directly to certain probiotic bacteria. Certain strains of *Lactobacillus,* for example, release surface-active components, which inhibit adhesion of pathogenic bacteria[38]. Thus, probiotic bacteria may protect epithelium by receptor competition, whereby probiotics compete with microbial pathogens for a limited number of receptors present on the surface epithelium.

In conclusion, the probiotic mixture, Bifico, is highly effective in reducing colitis in IL-10 KO mice. Furthermore, the present study revealed that Bifico treatment reduced colonic epithelial permeability, recovered normal expression and distribution of tight junction proteins, and protected against pathogenic bacterial invasion both *in vivo* and *in vitro*.

COMMENTS

Background

The human gut is colonized with a wide variety of microorganisms, including pathogenic, probiotic and commensal bacteria. *Bifidobacterium*, *Lactobacillus* and *Enterococcus faecalis* are probiotics with beneficial effects on maintenance therapy of human intestinal diseases. For example, oral treatment with specific probiotic bacteria can ameliorate inflammatory bowel disease. The colonization strategies using defined commensals or exogenous specific probiotic treatment may prevent host intestinal inflammation and ameliorate intestinal epithelial barrier function.

Research frontiers

The probiotic mixture, Bifico, is highly effective in the reducing colitis in interleukin (IL)-10 KO mice. Furthermore, Bifico treatment reduced colonic epithelial permeability, recovered normal expression and distribution of tight junction proteins, and protected against pathogenic bacterial invasion both *in vivo* and *in vitro*.

Innovations and breakthroughs

The probiotic mixture, Bifico had a direct effect on epithelial barrier function *in vivo* by reducing mucosal secretion of tumor necrosis factor-α and interferon-γ, and altering expression and distribution of tight junction proteins. Bifico exposure *in vitro* reduced bacterial invasion. Moreover, the effects of combined probiotics were more pronounced than those with single-strain probiotics.

Applications

By understanding the mechanism and effects of Bifico on the intestinal mucosal barrier, this study may represent a future strategy in the treatment of patients with colitis and increased intestinal permeability, such as ulcerative colitis, Crohn's disease and irritable bowel syndrome.

Peer review

The authors have performed a very good study of improving gastrointestinal mucosal biology in a colitis-like model and evaluating the effect of Bifico on the intestinal mucosal barrier, the expression and distribution of epithelial tight junction proteins, and secretion of inflammatory cytokines *in vivo* and *in vitro*.

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