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Tiantian Yang
China Agricultural University
Jing Zhang
China Agricultural University
Baseer Ahmad
Muhammad Nawaz Shareef University of Agriculture
Zaheer Abbas
China Agricultural University
Jinzhuan Li
China Agricultural University
Xiaohan Jiang
China Agricultural University
Haosen Zhang
China Agricultural University
Junyong Wang
China Agricultural University
Qiang Cheng
China Agricultural University
Rijun Zhang
China Agricultural University
Dayong Si
dayong@cau.edu.cn
China Agricultural University

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Clostridium butyricum strains XMC 312 and SWY 13 alleviate dextran sulfate sodium-induced colitis through restoration of intestinal barrier integrity and modulation of gut microbiota

Tiantian Yang[1,3], Jing Zhang[1], Baseer Ahmad[2], Zaheer Abbas[1,4], Jinzhuan Li[1], Xiaohan Jiang[1], Haosen Zhang[1], Junyong Wang[1], Qiang Cheng[1,5], Rijun Zhang[1], Dayong Si[1]*

[1]State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China

[2]Faculty of Veterinary and Animal Sciences, Muhammad Nawaz Shareef University of Agriculture, Multan 25000, Punjab, Pakistan

[3]Present address: Department of Comparative Biosciences, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA.

[4]Present address: School of Biomedical Sciences and Engineering, South China University of Technology, Guangzhou International Campus, Guangzhou 511442, China

[5]Present address: Poultry Science Institute, Chongqing Academy of Animal Sciences, Chongqing 402460, China

Corresponding author: Dayong Si

Email: dayong@cau.edu.cn

Abstract

Clostridium butyricum (C. butyricum) is a promising probiotic for maintaining intestinal homeostasis; however, its therapeutic efficacy is highly strain-dependent, and the biological

activities of newly isolated strains remain poorly characterized. Here, we investigated the protective efficacy and underlying mechanisms of two newly isolated *C. butyricum* strains (XMC 312 and SWY 13) in a dextran sulfate sodium (DSS)-induced murine colitis model by comprehensively evaluating intestinal barrier integrity, oxidative stress, immune responses, gut microbiota composition, and cecal short-chain fatty acid (SCFA) production. Both strains attenuated DSS-induced body weight loss, reduced disease activity index (DAI) scores, and improved colonic histopathological injury. Notably, SWY 13 was associated with increased goblet cell abundance and higher expression of mucin 2 (MUC2), ZO-1, and Claudin-1, whereas XMC 312 was mainly associated with increased Occludin and Claudin-1 expression. In parallel, both strains improved serum total antioxidant capacity (T-AOC) and mitigated the DSS-induced decline in serum immunoglobulin G (IgG). Gut microbiota profiling via 16S rRNA gene sequencing further revealed that XMC 312 and SWY 13 altered the DSS-induced gut dysbiosis, including enrichment of *Bifidobacterium* in both groups. Moreover, SWY 13 treatment was accompanied by increased *Lactobacillus*, decreased *Escherichia–Shigella*, and higher cecal acetate, propionate, and butyrate levels. Collectively, these findings demonstrate that both *C. butyricum* strains alleviate DSS-induced colitis through coordinated improvements in intestinal barrier integrity, host antioxidant defenses, gut microbial composition, and microbial metabolite production, highlighting their potential as probiotic candidates for further development to support intestinal health.

Keywords: *Clostridium butyricum*, Ulcerative colitis, Gut microbiota, Gut barrier

Highlights

- *C. butyricum* strains XMC 312 and SWY 13 ameliorated DSS-induced colitis in mice.
- Both strains increased the expression of colonic tight junction proteins in DSS-challenged mice.
- Both strains reshaped DSS-induced gut dysbiosis by enriching beneficial taxa and suppressing opportunistic pathogens.

1. Introduction

Inflammatory bowel disease (IBD), particularly ulcerative colitis (UC), is a chronic immune-mediated inflammatory disorder characterized by persistent mucosal inflammation, epithelial injury, and a continuously increasing global incidence, imposing a substantial healthcare burden worldwide [1]. Although the precise pathogenesis of IBD remains unclear, intestinal barrier dysfunction and gut microbiota dysbiosis are considered important contributors to disease onset and progression [2]. The intestinal barrier constitutes the first line of defense between luminal microorganisms and host tissues, integrating the mucus layer, epithelial cells, tight junctions, and mucosal immune components to restrict microbial translocation and maintain intestinal homeostasis [3]. The mucus layer, mainly formed by goblet cell-derived mucins such as MUC2, and epithelial tight junctions, including ZO-1, Occludin, and Claudins, are key components of the intestinal barrier [4, 5]. In IBD, inflammatory responses can impair these barrier elements by disrupting TJP expression and localization, increasing epithelial permeability, and facilitating luminal antigen or microbial translocation, which further aggravates mucosal inflammation and epithelial injury [6, 7].

Accumulating evidence indicates that gut microbiota dysbiosis is a hallmark of UC,

characterized by reduced microbial diversity, depletion of beneficial commensals, and expansion of opportunistic pathogens that collectively promote chronic intestinal inflammation [8, 9]. Under healthy conditions, the gut microbiota supports epithelial barrier integrity, produces beneficial metabolites, and contributes to mucosal immune homeostasis [3]. In UC, however, this protective microbial ecosystem is often disrupted, with enrichment of Enterobacteriaceae and reduction of beneficial SCFA-producing anaerobes [10, 11]. Such microbial imbalance can weaken epithelial barrier function, limit anti-inflammatory metabolite production, and sustain mucosal inflammation and tissue injury [12, 13]. Besides barrier disruption and gut microbiota dysbiosis, oxidative stress is increasingly recognized as an important contributor to colonic injury in colitis, reflecting excessive oxidant production and insufficient endogenous antioxidant defenses [14].

Given the central roles of gut microbial dysbiosis and epithelial barrier dysfunction in UC pathogenesis, microbial-targeted interventions, particularly probiotics, have emerged as promising therapeutic strategies for restoring intestinal homeostasis. Probiotics have been increasingly investigated as adjunctive microbiota-targeted approaches in IBD, especially UC, although their efficacy varies depending on the probiotic strain, dosage, host condition, disease stage, and treatment regimen [15]. Previous studies have reported that probiotic inverventions may alleviate intestinal inflammation, influence gut microbial homeostasis, and support mucosal barrier function through inhibition of pathogen colonization, modulation of host immune responses, and regulation of anti-inflammatory signaling pathways [16-18].

Among candidate probiotics, the spore-forming and butyrate-producing anaerobe *C. butyricum* has attracted considerable attention because of its capacity to regulate immune

responses, reinforce epithelial barrier integrity, and reshape gut microbial communities [19-22]. Previous studies on *C. butyricum* have reported attenuation of inflammatory responses, enhancement of antioxidant capacity, and maintenance of intestinal homeostasis [23, 24]. As a key SCFA, butyrate contributes to epithelial barrier preservation, mucosal immune regulation, and maintenance of gut microbial and mucus layer homeostasis [25, 26]. However, because the probiotic effects of *C. butyricum* may vary across strains, newly isolated animal-derived strains require individual evaluation, and information regarding their effects in experimental colitis remains limited [27-29]. A recent study showed that a giant panda-derived *C. butyricum* isolate alleviated DSS-induced colitis in mice, suggesting that animal-derived *C. butyricum* strains may represent a useful source of probiotic candidates for intestinal inflammation [30]. In our previous work, XMC 312 and SWY 13 were isolated from broiler feces and beef rumen fluid, respectively, and both exhibited antioxidant and anti-inflammatory activities in vitro (data not shown). However, the in vivo effects of these two newly isolated strains on colitis remain unclear.

Therefore, the study aimed to determine the protective effects and potential mechanisms of two newly isolated *C. butyricum* strains (XMC 312 and SWY 13) against DSS-induced colitis by comprehensively evaluating disease severity, intestinal barrier integrity, inflammatory responses, gut microbiota composition, and microbial metabolite production.

2. Materials and methods

2.1 Materials

DSS (molecular weight 36−50 kDa) was purchased from MP Biomedicals (California,

USA). Mouse ELISA kits were supplied by Beijing Silian Changyuan Biotechnology Co., Ltd. (Beijing, China). Standards of acetic acid, propionic acid, and butyric acid with a purity of ≥99% were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). All other analytical-grade reagents were purchased from Shanghai Chemicals Reagents Co., Ltd. (Shanghai, China). Protease K was sourced from Beyotime Biotechnology (Nanjing, China).

2.2 Preparation of *C. butyricum* culture

C. butyricum strains (XMC 312 and SWY 13) were originally isolated from broiler feces and rumen fluid from beef cattle, respectively. Fresh fecal samples were collected from broilers maintained at the teaching and research farm of the College of Animal Science and Technology, China Agricultural University, Beijing, China. Rumen fluid was collected via the rumen fistula from fistulated beef cattle maintained at Beijing Zhongdi Breeding Livestock Co., Ltd., Beijing, China. The identities of XMC 312 and SWY 13 were confirmed by 16S rRNA gene sequencing. Their 16S rRNA gene sequences are available in GenBank with accession numbers OQ435277.1 and OQ435302.1, respectively. Both strains were propagated anaerobically in Reinforced Clostridial medium (RCM) at 37 °C for 24 h and collected near the end of the logarithmic growth phase. RCM contained 3.0 g/L yeast extract, 10.0 g/L beef extract, 10.0 g/L peptone, 5.0 g/L glucose, 1.0 g/L soluble starch, 5.0 g/L sodium chloride, 3.0 g/L sodium acetate, and 0.5 g/L L-cysteine hydrochloride.

2.3 Experimental design and animal housing

Thirty-six male C57BL/6J mice, 6 weeks of age, were purchased from Beijing Vital River

Laboratory Animal Technology Co., Ltd. Animals were maintained under specific pathogen-free conditions at 22-25 °C and 55–60% relative humidity on a 12-h light/12-h dark cycle. Mice had ad libitum access to sterilized water and a sterilized standard chow diet purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Diet composition is provided in Table 1. Following a 7-day acclimation period, mice were randomly allocated to six groups with similar initial mean body weights (n=6/group): untreated control (CON), XMC 312, SWY 13, DSS, XMC 312+DSS, and SWY 13+DSS.

From day 0 to day 28, mice in the CON and DSS groups received daily oral gavage with distilled water. During the same period, mice in the XMC 312 and XMC 312+DSS groups received *C. butyricum* XMC 312 by daily oral gavage, while mice in the SWY 13 and SWY 13+DSS groups received *C. butyricum* SWY 13 by daily oral gavage. The bacterial suspensions were administered at a concentration of 10^8 CFU/mL in a volume of 200 μL per mouse per day, corresponding to 2×10^7 CFU per mouse per day. The dose and regimen were based on previous murine studies of *C. butyricum* [31].

To induce colitis, mice in the DSS, XMC 312+DSS, and SWY 13+DSS groups were given 2% DSS (w/v) in drinking water from day 21 to day 28. Thus, bacterial treatment was initiated 21 days before DSS exposure and continued throughout the 7-day DSS administration period. On day 29, mice were anesthetized with isoflurane, followed by collection of blood from the retro-orbital sinus. Cervical dislocation was then performed for euthanasia, and death was verified before tissues were harvested. The colons were collected and photographed, and colon length was measured. A 0.5-cm portion of the distal colon was rinsed with PBS and immersed in 4% paraformaldehyde (w/v) at 4 °C before routine dehydration and paraffin embedding. The

rest of the colonic tissue was immediately stored at −80 °C for subsequent analyses.

2.4 DAI and Histopathological Inflammation Scores

The DAI score was assessed daily based on body weight loss, stool consistency, and fecal bleeding, following a previously described method [32]. Scoring criteria were as follows: weight loss (0: none, 1:1−10%, 2: 11−15%, 3: 16−20%, 4: >20%); stool consistency (0−1: normal, 2−3: loose stool, 4: diarrhea); and fecal blood content (0−1: normal, 2−3: occult blood, 4: gross bleeding).

Histopathological inflammation scores were determined with slight modifications from established protocols [32]. Specifically, histological assessment included edema, glandular dilation, epithelial injury, crypt destruction, and inflammatory-cell infiltration. Histopathological lesions were graded on a 0–5 scale: 0, no detectable lesion; 1, focal mild injury; 2, focal moderate injury; 3, focal severe lesion; 4, extensive moderate lesion; and 5, extensive severe lesion. Colon tissues were harvested for length measurement and histological assessment by hematoxylin and eosin (H&E) staining. For histological examination, distal colon samples fixed in 4% paraformaldehyde were dehydrated through a graded ethanol series, embedded in paraffin, sectioned, deparaffinized, and stained with H&E.

2.5 Assessment of Goblet Cell Density in the Colon by PAS Staining

Goblet cells in deparaffinized colon sections were visualized using a commercial periodic acid–Schiff (PAS) staining kit (Servicebio, Wuhan, China). Briefly, sections were incubated in PAS solution B for 10-15 min, followed by PAS solution A for 25-30 min in the dark. Sections

were then treated with PAS solution C for 30 s and rinsed with tap water, followed by sequential treatment with hydrochloric acid and ammonia. After dehydration, five randomly selected non-overlapping fields from a one colon section per mouse were evaluated. Mucosal length in each field was measured using ImageJ software (version 1.54; National Institutes of Health, Bethesda, MD, USA), and the number of PAS-positive goblet cells was normalized to the corresponding mucosal length. The mean value from the five fields was used as the representative value for each animal.

2.6 Serum immunoglobulin and antioxidant ability.

Blood was collected from the orbital venous plexus and centrifuged at 2,500 g for 10 min at 4 °C to obtain serum. The levels of immunoglobulins (IgA, IgG, IgM) were measured using ELISA kits (Beijing Siliang Changyuan Biotechnology Co., Ltd., Beijing, China). Serum T-AOC, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) concentration were assayed with commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following the supplier's protocols. The absorbance of each assay reaction was measured at the assay-specific wavelength using a K5600C microvolume UV–Vis spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China).

2.7 Apoptosis assay

Apoptosis in colonic sections was detected using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (Servicebio, Wuhan, China). TUNEL labeling was performed by exposing colon sections to a reaction mixture of TdT enzyme, dUTP, and reaction

buffer at a 1:5:50 ratio for 2 h at 37 °C, as specified by the manufacturer. 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was subsequently applied as a nuclear counterstain. After three washes with PBS, images were captured in the DAPI and FITC channels using a Nikon Eclipse C1 upright fluorescence microscope equipped with a Nikon DS-U3 imaging system (Nikon, Tokyo, Japan). For each animal, the percentage of TUNEL-positive cells was quantified in five randomly selected, non-overlapping microscopic fields from one colon section. The mean percentage across the five fields was used as the animal-level value for statistical analysis.

2.8 Immunohistochemical Evaluation of Colonic Inflammation and Barrier Proteins

Paraffin-embedded tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide, followed by blocking with 3% bovine serum albumin to reduce non-specific binding. Sections were then incubated overnight at 4 °C with primary antibodies (Servicebio, Wuhan, China): anti-MPO (GB11224, 1:200), anti-MUC2 (GB11344, 1:500), anti-ZO-1 (GB111981, 1:200), anti-occludin (GB111401, 1:500), and anti-claudin1 (GB14066, 1:100). After washing, sections were incubated with HRP-conjugated secondary antibodies at room temperature for 30 min. Following chromogenic development and hematoxylin counterstaining, slides were dehydrated, cleared, mounted, and examined by bright-field microscopy. For each marker, ImageJ software (version 1.54; National Institutes of Health, Bethesda, MD, USA) was used to determine the immunopositive area as a percentage of the total tissue area examined. Measurements were obtained from five randomly selected, non-

overlapping fields from one colon section per mouse and averaged to generate a representative animal-level value for statistical analysis. Bright-field images of H&E-, PAS-, and immunohistochemically stained colon sections were captured using a Nikon Eclipse E100 light microscope equipped with a Nikon DS-U3 imaging system (Nikon, Tokyo, Japan).

2.9 Gene Expression Analysis in Intestinal Tissues

Colon tissue was homogenized in TRIzol reagent (Vazyme Biotech Co., Ltd., Nanjing, China) for total RNA isolation. Because residual DSS can interfere with downstream analysis, RNA was further cleaned by lithium chloride precipitation according to Viennois et al. [33]. Purified RNA was converted to cDNA with HiScript RT SuperMix for qPCR (Vazyme Biotech Co., Ltd., Nanjing, China). Quantitative real-time PCR was performed using 2× M5 HiPer SYBR Premix EsTaq (Mei5 Biotechnology Co., Ltd., Beijing, China) on a LightCycler 96 Real-Time PCR System (Roche, Switzerland). Relative mRNA expression levels were quantified using the $2^{-\Delta\Delta}$Ct method. Each cDNA sample was analyzed in triplicate, and the primer sequences are provided in Table 2.

2.10 Gas chromatography for SCFAs analysis

SCFAs in mouse cecal contents were analyzed using a GC-2010 Plus gas chromatograph equipped with a flame ionization detector (Shimadzu Corporation, Kyoto, Japan). Briefly, 150 mg of frozen cecal content was suspended in distilled water and vortexed for 10 min, followed by incubation on ice for 20 min. The mixture was centrifuged at 14,000 g for 10 min, and the supernatant was collected. After the initial centrifugation, the aqueous phase was recovered

and combined with 25% (w/v) metaphosphoric acid at a 5:1 supernatant-to-acid volume ratio. Samples were maintained at 4 °C for 30 min and centrifuged again at 14,000 g for 10 min. The resulting supernatant was appropriately diluted, supplemented with crotonic acid as the internal standard, passed through a 0.22-μm membrane, and subjected to GC analysis. Chromatographic separation was achieved on a DB-23 capillary column (30 m × 0.32 mm i.d. × 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). The oven maintained at 180 °C for 10 min, increased to 220 °C at 4 °C/min and held for 15 min, and then increased to 250 °C at 3 °C/min with a final 30-min hold. Injector and detector temperatures were maintained at 250 °C and 280 °C, respectively. SCFAs were quantified using standard curves of mixed SCFA standards with internal standard correction.

2.11 16S rRNA gene sequencing for microbiota analysis

To investigate gut microbiota alterations induced by *C. butyricum* pretreatment, six colonic content samples from each group were subjected to 16S rRNA gene amplicon sequencing. Microbial DNA was isolated with the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, USA). After amplification of the V3-V4 region, purified PCR products were sequenced using the Illumina MiSeq platform. Sequencing data from this experiment are available in the NCBI Sequence Read Archive under BioProject PRJNA1426739.

Demultiplexing and quality control of raw reads were carried out with fastp v0.19.6 [34], followed by assembly of paired-end sequences using FLASH v1.2.7 [35]. Sequences were excluded when they were of low quality, shorter than 50 bp, contained ambiguous nucleotides, or failed to merge successfully. The optimized sequences were clustered into operational

taxonomic units (OTUs) at 97% sequence similarity using UPARSE v7.1 [36], and the most abundant sequence in each OTU was selected as the representative sequence.

Taxonomic assignment was performed using the RDP classifier v2.11 [37] against the Silva 16S rRNA gene database v138. Bioinformatic analyses were conducted on the Majorbio Cloud Platform (https://cloud.majorbio.com). Alpha diversity indices, including Chao and Shannon indices, were calculated using mothur [38]. Beta diversity was analyzed using QIIME 2. Overall similarities in microbial community structure were visualized by principal component analysis (PCA) using Euclidean distances, whereas partial least squares discriminant analysis (PLS-DA) was applied to examine separation among experimental groups. Differentially abundant taxa were identified using linear discriminant analysis (LDA) effect size (LEfSe) [39], with an LDA score > 4 considered significant.

2.12 Statistical analysis

Data are presented as mean ± SEM. For single-time-point variables, differences among groups were analyzed using one-way ANOVA followed by Tukey's HSD post hoc test. Body weight and DAI were analyzed by two-way repeated-measures ANOVA, with treatment as the between-subject factor and time as the within-subject factor, followed by post hoc multiple-comparison tests at each time point. All statistical tests were carried out in IBM SPSS Statistics 26.0 (IBM SPSS Statistics, Chicago, IL, USA). A probability value below 0.05 was considered statistically significant. Box plots were generated using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1 Pretreatment with XMC 312 and SWY 13 alleviated DSS-induced colitis

Mice were pretreated with *C. butyricum* strains XMC 312 and SWY 13 for 21 days to evaluate their protective effects against DSS-induced colitis (Fig. 1A). Acute colitis was induced by 2% DSS in drinking water for 7 days, which led to body weight loss, diarrhea, bloody stools, increased DAI, and shortened colon length. Compared with the DSS group, mice in the XMC 312+DSS group showed significantly higher body weight on days 6–7, lower DAI on days 4–5, and increased colon length (Fig. 1B–E). Likewise, mice in the SWY 13+DSS group exhibited reduced weight loss on days 5–7, lower DAI on days 3–7, and longer colon length than those in the DSS group (Fig. 1B–E). No significant differences were observed in liver, spleen, and kidney indices among groups (Fig. 1F–H). Collectively, these findings indicate that XMC 312 and SWY 13 pretreatment markedly mitigates DSS-induced colitis.

3.2 XMC312 and SWY13 pretreatment attenuated DSS-induced colonic damage

HE staining showed that XMC312 and SWY13 pretreatment ameliorated DSS-induced colon injury, as evidenced by reduced epithelial damage, crypt loss, inflammatory cell infiltration, and loss of glandular epithelial cells (Fig. 2A). Accordingly, mice in the XMC 312+DSS group exhibited significantly lower histological damage scores than those in the DSS group (Fig. 2B). Colonic myeloperoxidase (MPO) expression, a marker of neutrophil infiltration, was assessed by immunohistochemical staining. DSS exposure significantly increased MPO-positive staining compared with the CON group (Fig. 2C). Compared with the DSS group, MPO-positive staining showed a decreasing trend in the SWY 13 + DSS group (*P*

= 0.061) (Fig. 2D). Moreover, TUNEL staining showed that DSS challenge increased epithelial apoptosis in the colon (Fig. 2E). Quantitative analysis further confirmed the number of TUNEL-positive cells was significantly reduced in the SWY13 + DSS group compared with the DSS group (Fig. 2F).

3.3 XMC312 and SWY13 pretreatment improved serum oxidative indexes and immunoglobulin levels

DSS treatment decreased serum T-AOC and SOD activity compared with the control group (Fig. 3A–B). Pretreatment with XMC 312 or SWY 13 significantly increased T-AOC in DSS-challenged mice (Fig. 3A). DSS also reduced serum IgG levels, and this reduction was attenuated by pretreatment with either strain (Fig. 3E). No significant differences were observed in serum MDA, IgA, and IgM levels among the groups (Fig. 3C, D, F).

3.4 XMC312 and SWY13 pretreatment improved DSS-induced colonic barrier dysfunction

The colonic mucus layer constitutes the first line of defense against luminal toxins and plays a critical role in protection against colitis [40]. Goblet cell-derived mucins (MUCs) are key components of this barrier. PAS staining showed that DSS treatment caused goblet cell depletion in the colon, whereas pretreatment with strain SWY 13 ameliorated goblet cell loss (Fig. 4A–B). Consistently, immunohistochemical staining revealed reduced MUC2 immunoreactivity in DSS-treated mice compared with the control group (Fig. 4C). Compared with the DSS group, MUC2 expression showed an increasing trend in the XMC 312 + DSS group, although the difference was not statistically significant (P = 0.072). In contrast, SWY

13 pretreatment significantly restored MUC2 expression in DSS-challenged mice (Fig. 4C–D).

RT–qPCR was further performed to quantify mRNA levels of mucin genes (*Muc2*, *Muc4*), tight junction-related genes (*Tjp3*, *Ocln*, *Cldn2*, and *Cldn5*), inflammatory markers (*Il1b*, *Il4*, *Il10*, *Tnf, Nfkb, Cox2*), and nuclear factor erythroid 2-related factor 2 (*Nrf2*) in colonic tissue. DSS treatment altered the expression of several of these genes (Fig. 5). Among DSS-challenged mice, pretreatment with XMC 312 significantly elevated the transcript levels of *Muc2*, *Muc4*, and *Tjp3*, and *Ocln* (Fig. 5A–D), whereas SWY 13 pretreatment significantly increased *Cldn5* expression (Fig. 5F). Regarding inflammatory markers, SWY 13 significantly reduced *Il1b* mRNA levels (Fig. 5G), while XMC 312 significantly decreased *Il4* mRNA levels (Fig. 5H). Both strains markedly increased *Il10* mRNA levels compared with the DSS group (Fig. 5I). In addition, SWY 13 pretreatment significantly downregulated *Cox2* and upregulated *Nrf2* mRNA levels relative to DSS alone (Fig. 5L–M). In the absence of DSS, SWY 13 alone also significantly increased *Muc2* mRNA levels relative to the CON group (Fig. 5A).

Immunohistochemical staining of tight junction proteins (TJPs) showed greater ZO-1, Occludin, and Claudin-1 expression in DSS-challenged mice pretreated with XMC 312, whereas SWY 13 increased ZO-1 and Claudin-1 expression (Fig. 4E–J).

3.5 XMC312 and SWY13 pretreatment modulated gut microbiota composition in DSS-induced colitis

Dysbiosis of the gut microbiota is strongly associated with the pathogenesis of IBD. To investigate changes in the colonic microbiota after pretreatment with XMC 312 or SWY 13, we analyzed the composition and diversity. Alpha diversity analysis showed that, relative to

DSS alone, the SWY 13+DSS group displayed a lower Shannon index and a higher Simpson index, indicating an altered pattern in alpha diversity (Fig. 6B–C). PCA placed the CON, DSS, and strain-treated samples in distinct regions of the ordination space, indicating differences in overall community structure (Fig. 6D). PLS-DA at the genus level also showed distinct clustering patterns among groups (Fig. 6E).

LEfSe analysis revealed distinct microbial signatures among groups (Fig. 6F–G). The CON group showed enrichment of Lachnospiraceae and related taxa (Fig. 6F). In the absence of DSS, XMC 312 was characterized by enrichment of Actinobacteriota-lineage taxa, particularly *Bifidobacterium*, whereas SWY 13 was characterized by enrichment of *Lactobacillus* and higher-level taxa within Lactobacillales (Fig. 6F). DSS treatment was associated with expansion of Proteobacteria, notably Gammaproteobacteria, Enterobacterales, Enterobacteriaceae, *Escherichia–Shigella,* and *Enterococcus* (Fig. 6G). Under DSS challenge, the two strain-treated groups showed taxonomic profiles distinct from that of the DSS group: XMC 312+DSS was enriched in Alcaligenaceae and *Achromobacter*, whereas SWY 13+DSS was enriched in Actinobacteriota- and *Bifidobacterium*-related taxa (Fig. 6G).

At the phylum level, Firmicutes, Actinobacteriota, and Bacteroidota predominated across groups (Fig. 7A). In the absence of DSS, pretreatment with either strain significantly increased the relative abundance of Actinobacteriota (Fig. 7C). SWY 13 alone also significantly increased the relative abundance of Proteobacteria (Fig. 7F). DSS treatment significantly reduced Actinobacteriota and increased Proteobacteria (Fig. 7C, 7F). Under DSS challenge, Actinobacteriota was more abundant in both strain-pretreated groups than in mice receiving DSS alone, whereas the abundance of Proteobacteria was lower following SWY 13 pretreatment (Fig. 7C, 7F).

The genus-level community was dominated primarily by *Dubosiella, Lactobacillus,* and *Turicibacter* (Fig. 7G). Compared with the CON group, SWY 13 increased both *Lactobacillus* and *Bifidobacterium*, while XMC 312 significantly increased *Bifidobacterium* (Fig. 7I, 7K). DSS treatment significantly decreased *Enterorhabdus* and increased *Turicibacter*, *Escherichia–Shigella, Enterococcus,* and *Bacteroides* (Fig. 7J, 7L–O). Both strain-pretreated DSS groups contained significantly more *Bifidobacterium* than the DSS-only group (Fig. 7K). *Escherichia–Shigella* tended to decrease after XMC 312 pretreatment (P = 0.059) and was significantly lower after SWY 13 pretreatment (Fig. 7M). *Enterococcus* and *Bacteroides* were also reduced in the SWY 13+DSS group (Fig. 7N, 7O). These findings show that pretreatment with the two strains was accompanied by distinct changes in gut microbiota composition, including increases in beneficial taxa under DSS challenge.

3.6 SWY13 pretreatment increased cecal contents of SCFAs

The metabolic profile of cecal contents is closely related to gut microbiota composition. Cecal SCFA concentrations were measured to assess changes in microbial metabolic output. DSS exposure reduced cecal acetate, propionate, butyrate, and total SCFAs (Fig. 8A–D). SWY 13 pretreatment significantly increased all four parameters compared with the DSS group. In addition, XMC 312 pretreatment showed upward trends in propionate (P = 0.066), butyrate (P = 0.095), and total SCFAs (P = 0.088), none of which reached statistical significance.

4. Discussion

UC has become a major global health challenge, creating an urgent need for safe and

effective microbiota-targeted therapeutic strategies [41]. Recent studies suggest that regulation of the epithelial barrier, immune responses, and gut microbiota represents an effective therapeutic strategy [42]. *C. butyricum* is a butyrate-producing bacterium, has been widely recognized for its anti-inflammatory and barrier-protective properties [43-45], but specific effects of individual *C. butyricum* strains remain unclear. In general, an ideal probiotic should stably colonize the host and confer physiological benefits through immunomodulation, attenuation of inflammation, and regulation of the gut microbiota [46]. Microbial metabolites, including lactic acid and butyric acid, are also important mediators of intestinal and systemic homeostasis [45, 47, 48]. Previous studies have shown that *C. butyricum* can modulate gut microbial composition, reshape intestinal metabolite profiles, and promote the expression of mucin and TJP expression in intestinal epithelial cells (IECs) [49-52].

In the present study, DSS exposure induced the expected acute-colitis phenotype, characterized by loss of body mass, diarrhea, hematochezia, elevated DAI scores, and shortening of the colon. Pretreatment with XMC 312 or SWY 13 reduced disease severity, as reflected by lower DAI scores, mitigated hematochezia, and improved histopathological damage. The two strains altered tight junction-related gene expression and other colonic barrier-associated parameters. Overall, both XMC 312 and SWY 13 alleviated DSS-induced colitis in mice and were accompanied by changes in gut microbiota composition.

Oxidative stress is recognized as a central pathological mechanism contributing to epithelial injury, barrier dysfunction, and sustained inflammation during UC. Excessive reactive oxygen species (ROS) can damage biomolecules in IECs and overwhelm endogenous antioxidant defenses, thereby compromising membrane integrity and barrier function [53].

Therefore, restoration of endogenous antioxidant defenses represents an important therapeutic strategy for limiting intestinal tissue injury and disease progression. Prior studies indicate that studies on *C. butyricum* have reported effects on antioxidant defenses, including increases in enzymes like glutathione peroxidase, catalase, and SOD [24, 54], which is consistent with the increase in serum T-AOC observed after pretreatment with XMC 312 or SWY 13 in the present study. Moreover, inflammation-driven ROS can activate NF-κB signaling and induce pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-6, thereby amplifying mucosal inflammation [53]. In contrast, *C. butyricum* has also been shown to affect pro-inflammatory cytokine gene expression and upregulate *Il10*. Consistently, serum IgG levels were higher in strains–pretreated mice in the present study, whereas IgA and IgM were not significantly changed.

The Nrf2 pathway is a central cellular defense against oxidative injury, largely through induction of cytoprotective enzymes such as heme oxygenase-1 (HO-1) [55]. HO-1 can enhance antioxidant capacity and dampen inflammatory signaling, and its induction has been associated with reduced cyclooxygenase-2 (COX-2) expression in inflammatory contexts [56]. In the present study, SWY 13 was associated with increased *Nrf2* mRNA and decreased *Cox2* mRNA, although the underlying mechanisms were not directly examined. These findings indicate that SWY 13 affected selected oxidative stress- and inflammation-related indicators in the present model.

Preservation of intestinal barrier integrity represents one of the principal mechanisms through which probiotics protect against experimental colitis. The intestinal barrier consists of the mucus layers and epithelial junctional complexes that collectively restrict pathogen

invasion and maintain mucosal homeostasis [57]. MUC2, secreted predominantly by goblet cells, is a major structural component of the mucus layer and a critical determinant of epithelial integrity [58, 59]. In the present study, DSS caused mucus thinning and reduced mucin production, whereas SWY 13 supplementation preserved goblet cell abundance and increased MUC2 expression, consistent with reports showing that probiotics reinforce the mucus barrier by maintaining goblet cells and promoting MUC expression [60]. Interestingly, XMC 312 increased *Muc2* and *Muc4* mRNA levels, suggesting altered expression of mucin-related genes, potentially mediated by butyrate produced by *C. butyricum* [61].

TJPs, including Occludin, Claudin, and ZO-1, are essential for regulating paracellular permeability and maintaining gut barrier integrity [6]. In the present study, DSS impaired colonic tight junctions, as indicated by reduced expression of ZO-1, Occludin, and Claudin-1. Disruption of these junctions is closely linked to barrier leakage and tissue injury in IBD [62]. Previous studies have shown that *C. butyricum* upregulates gut barrier proteins and promotes epithelial integrity [30] and that its bacteriocins can attenuate endotoxin-induced apoptosis and epithelial damage [63]. Importantly, TJP regulation is closely shaped by the inflammatory milieu. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-1β disrupt tight junction organization and increase epithelial permeability, whereas anti-inflammatory mediators, including IL-10 and transforming growth factor-β (TGF-β), help preserve barrier function by suppressing inflammation and promoting epithelial repair [6, 64]. Consistently, XMC 312 decreased *Il1b* expression, SWY 13 downregulated *Il4*, and both strains upregulated *Il10*, indicating a shift toward a less inflammatory cytokine profile that may favor epithelial homeostasis. Collectively, these findings indicate that restoration of

the mucus layer together with reinforcement of epithelial tight junctions constitutes a major mechanism underlying the protective effects of XMC312 and SWY13 against DSS-induced intestinal injury.

IBD is commonly associated with gut microbial dysbiosis, typically characterized by reduced diversity and a shift from beneficial taxa such as *Clostridium* and *Bacteroides* toward an expansion of Proteobacteria and opportunistic genera such as *Escherichia–Shigella and Enterococcus* [65, 66]. Inflammation can further favor facultative anaerobes by increasing luminal oxygen availability, thereby exacerbating microbial imbalance. Previous studies have reported that *C. butyricum* can modulate gut microbiota composition, including enrichment of SCFA-producing bacteria, such as *Lachnospiraceae* and reduction of potentially detrimental genera such as *Ruminiclostridium* [67, 68]. In addition, *C. butyricum*-derived extracellular vesicles or co-administration with fecal microbiota transplantation has been associated with changes in microbial communities, SCFA recovery, and alleviation of colitis symptoms in previous studies [69, 70]. In line with this, *Proteobacteria* overgrowth, particularly *Escherichia–Shigella*, has been linked to elevated inflammatory markers such as MPO, IL-6, and TNF-α, while *Lactobacillus* and *Bifidobacterium* have been associated with mucosal repair and immune homeostasis [71, 72].

Consistent with previous reports, both *C. butyricum* strains substantially remodeled the DSS-disrupted gut microbiota by suppressing opportunistic pathogens while enriching beneficial bacterial populations associated with intestinal health, as reflected by reduced relative abundance of potentially pathogenic taxa and enrichment of beneficial bacteria, XMC 312 mainly increased *Bifidobacterium*, while SWY 13 increased both *Lactobacillus* and

Bifidobacterium. These microbial alterations coincided with improved epithelial barrier integrity, reduced inflammatory responses, and enhanced intestinal homeostasis, suggesting that microbiota remodeling contributed to the observed therapeutic benefits. However, the causal relationships among microbiota changes, inflammatory responses, and barrier protection should be further investigated. The reduced MUC2 level observed in the XMC 312 group may reflect mucin-associated microbial adaptation, possibly related to the enrichment of *Bifidobacterium*, which has been reported to utilize mucin-derived glycans [73, 74].

SCFAs, particularly acetate, propionate, and butyrate, are key microbial metabolites that serve as critical mediators linking gut microbiota composition with epithelial barrier function, immune regulation, and intestinal homeostasis [13, 75]. SCFAs have been reported to enhance epithelial barrier function and modulate immune responses, in part through activation of G-protein-coupled receptors such as FFAR2/FFAR3 and, particularly for butyrate and propionate, inhibition of histone deacetylases [61]. Dysregulated SCFA metabolism is commonly observed in IBD. For example, UC patients often exhibit reduced butyrate-mediated immunoregulatory activity [76]. In the present study, DSS markedly reduced cecal SCFA levels, whereas SWY 13 significantly restored acetate, propionate, and butyrate. In the XMC 312 group, propionate and butyrate levels tended to recover compared with the DSS group. These SCFA changes were observed together with improved epithelial integrity and reduced inflammatory injury, but the present study did not directly determine whether SCFAs mediated these protective effects. Previous studies have suggested that propionate can suppress NLRP3 inflammasome activation in DSS-induced colitis through pathways involving PI3K/AKT signaling [77], while butyrate can reinforce barrier function by upregulating TJPs [78]. Collectively, these findings suggest

that microbiota remodeling and enhanced SCFA production may represent key mechanisms through which *C. butyricum* strains preserve epithelial barrier integrity and attenuate intestinal inflammation during DSS-induced colitis. Whether these microbial and metabolic shifts are causally responsible for the observed protection remains to be determined.

Several limitations of the present study should be acknowledged. First, the causal relationships among gut microbiota modulation, SCFA alterations, and intestinal barrier protection were not directly established. Second, although SCFAs were quantified in vivo, broader metabolomic characterization of intestinal contents, including strain-derived metabolites, could help identify additional mediators associated with mucosal protection. Third, efficacy was examined only in the DSS mouse model; confirmation in additional experimental models will therefore be necessary. Finally, comprehensive strain-level genomic and phenotypic safety assessments were not performed. Future studies should evaluate toxin- and virulence-associated genes, antimicrobial resistance determinants, phenotypic antimicrobial susceptibility, and other relevant safety characteristics, together with efficacy and optimal dosage, to further establish the safety and probiotic potential of these strains.

5. Conclusion

In conclusion, this study demonstrates the protective potential of two newly isolated *C. butyricum* strains, XMC312 and SWY13, against DSS-induced colitis. Their protective effects were associated with improvements in intestinal barrier integrity, host antioxidant defenses, gut microbiota composition, and cecal SCFA profiles. These findings support the further evaluation of XMC 312 and SWY 13 strain-level probiotic candidates for modulating intestinal

inflammation and maintaining gut homeostasis. However, the causal relationships among microbiota modulation, SCFA alterations, and mucosal protection remain to be established. Comprehensive strain-level safety assessments and validation in additional in vivo models are also required to further establish their probiotic potential.

List of abbreviations

Clostridium butyricum C. butyricum

Dextran sulfate sodium DSS

Cecal short-chain fatty acid SCFA

Disease activity index DAI

Mucin 2 MUC2

Total antioxidant capacity T-AOC

Immunoglobulin G IgG

Inflammatory bowel disease IBD

Ulcerative colitis UC

Reinforced Clostridial medium RCM

Hematoxylin and eosin H&E

Periodic acid–Schiff PAS

Superoxide dismutase SOD

Malondialdehyde MDA

Terminal deoxynucleotidyl transferase dUTP nick end labeling TUNEL

4′,6-diamidino-2-phenylindole dihydrochloride DAPI

Operational taxonomic units OTUs

Principal component analysis PCA

Partial least squares discriminant analysis PLS-DA

Linear discriminant analysis LDA

Linear discriminant analysis effect size LEfSe

Myeloperoxidase MPO

Nuclear factor erythroid 2-related factor 2 Nrf2

Tight junction proteins TJPs

Intestinal epithelial cells IECs

Reactive oxygen species ROS

Interleukin-1β IL-1β

Heme oxygenase-1 HO-1

Cyclooxygenase-2 COX-2

Tumor necrosis factor-α TNF-α

Interferon-γ IFN-γ

Transforming growth factor-β TGF-β

Ethics approval and consent to participate

The animal experiment was approved by the Animal Care and Use Committee of China Agricultural University (Approval No. AW13012222-1-3). Permission to collect the broiler fecal samples was obtained from the responsible teaching and research farm of China Agricultural University, and permission to collect rumen fluid from the fistulated cattle was

obtained from Beijing Zhongdi Breeding Livestock Co., Ltd., the owner of the animals.

Consent for publication

Not applicable

Availability of data and materials

The raw 16S rRNA gene sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA1426739. The remaining data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

TY contributed to conceptualization, methodology, investigation, data curation, and writing the original draft. JZ contributed to methodology and writing—review and editing. BA contributed to formal analysis and writing—review and editing. ZA contributed to data curation

and writing—review and editing. JL contributed to methodology and formal analysis. XJ contributed to resources and software. HZ contributed to formal analysis. JW contributed to formal analysis and data curation. QC contributed to resources and investigation. RZ contributed to project administration and supervision. DS contributed to conceptualization, project administration, supervision, funding acquisition, and writing—review and editing. All authors read and approved the final manuscript.

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Figure legends

Fig. 1 Pretreatment with XMC 312 and SWY 13 alleviates DSS-induced acute colitis in mice. (A) Schematic diagram of the experimental timeline. (B) Changes in body weight following DSS treatment. (C) DAI scores in mice. (D) Representative images of mouse colons. (E) Comparison of colonic length (cm). Organ indices of the liver (F), spleen (G), and kidney (H). Data are presented as mean ± SEM (n = 6 mice per group). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared to the CON group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the DSS group.

Fig. 2 Pretreatment with XMC 312 and SWY 13 alleviates DSS-induced colonic histological damage. (A) Representative H&E-stained sections showing histological morphology. Scale bar: 50 μm. (B) Histological scoring of colonic tissue. (C) Representative immunohistochemical staining of MPO in colon sections. Scale bar: 100 μm. (D) Quantification of MPO-positive area (%). (E) Representative fluorescent micrographs of TUNEL-positive cells (green) and DAPI-stained nuclei (blue) in colonic sections. Scale bar: 100 μm. (F) Quantification of TUNEL-positive cells (%). Data are presented as mean ± SEM (n = 6 mice per group). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared to the CON group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the DSS group.

Fig. 3 Effect of XMC 312 and SWY 13 pretreatment on serum oxidative stress markers and immunoglobulin levels in mice. (A-F) Serum levels of T-AOC, SOD, MDA, IgA, IgG, and IgM. Data are presented as mean ± SEM (n = 6 mice per group). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared to the CON group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the DSS group.

Fig. 4 Pretreatment with XMC 312 and SWY 13 protects the colonic barrier in DSS-induced colitis. (A) Representative images of PAS-stained goblet cells in the colonic epithelium. Scale bar: 50 μm. (B) Quantification of PAS-positive goblet cells per unit length of colonic mucosa. (C) Representative immunohistochemical staining of MUC2. Scale bar: 100 μm. (D) Quantification of MUC2-positive area (%). (E, G, I) Representative immunohistochemical staining of ZO-1 (E), Occludin (G), and Claudin-1 (I). Scale bar: 100 μm. (F, H, J) Quantification of ZO-1-positive (F), Occludin-positive (H), and Claudin-1-positive (J) positive area (%). Data are presented as mean ± SEM (n = 6 mice per group). $^{\#}P<0.05$, $^{\#\#}P<0.01$,

$^{###}P$<0.001, compared to the CON group. *P<0.05, **P<0.01, ***P<0.001 compared with the DSS group.

Fig. 5 Effects of XMC 312 and SWY 13 pretreatment on colonic mRNA levels of mucins, TJPs, inflammatory cytokines, and oxidative stress-related factors. (A-M) Relative transcript levels of *Muc2*, *Muc4*, *Tjp3*, *Ocln*, *Cldn2*, *Cldn5, Il1b*, *Il4*, *Il10*, *Tnf*, *Nfkb*, *Cox2* and *Nrf2* in colon tissue. Data are presented as mean ± SEM (n = 6 mice per group). $^{#}P$<0.05, $^{##}P$<0.01, $^{###}P$<0.001, compared to the CON group. *P<0.05, **P<0.01, ***P<0.001 compared with the DSS group.

Fig. 6 Pretreatment with XMC 312 and SWY 13 alters gut microbiota composition in DSS-induced colitis. (A-C) Alpha diversity indices of colonic microbiota: Chao, Shannon, and Simpson indices. (D, E) PCA plot and PLS-DA based on genus-level microbial composition. (F, G) LEfSe analysis of differentially abundant microbial taxa. Data are presented as mean ± SEM (n = 6 mice per group). $^{#}P$<0.05, $^{##}P$<0.01, $^{###}P$<0.001, compared to the CON group. *P<0.05, **P<0.01, ***P<0.001 compared with the DSS group.

Fig. 7 Effect of XMC 312 and SWY 13 pretreatment on the relative abundances of predominant differential bacteria at the phylum and genus levels. (A–F) Relative abundances of major bacterial phyla. (G–O) Relative abundances of major bacterial genera. Data are presented as mean ± SEM (n = 6 mice per group). $^{#}P$<0.05, $^{##}P$<0.01, $^{###}P$<0.001, compared to the CON group. *P<0.05, **P<0.01, ***P<0.001 compared with the DSS group.

Fig. 8 Effect of XMC 312 and SWY 13 pretreatment on cecal SCFA levels. (A-D) Concentrations of acetic acid, propionic acid, butyric acid, and total SCFAs in the cecal contents. Data are presented as mean ± SEM (n = 6 mice per group). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared to the CON group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the DSS group.

Tables

Table 1. Nutrient composition of the standard chow diet

Nutrient	Content (%)
Nitrogen-free extract	50.5
Crude protein	23.90
Crude fat	5.60
Crude fiber	4.40
Crude ash	7.20
Calcium	1.33
Total phosphorus	0.96

Note: Values were provided by the manufacturer.

Table 2. Primer sequences of the target genes used in this study

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)

Gapdh	GTGAAGGTCGGTGTGAACGGATT	GGTCTCGCTCCTGGAAGATGGT
Il1b	AATCTCGCAGCAGCACATCAACA	ACACCAGCAGGTTATCATCATCATCC
Il4	GCTAGTTGTCATCCTGCTCTTCTTTCT	CTGTGGTGTTCTTCGTTGCTGTGA
Il10	GACAACATACTGCTAACCGACTCCTTAA	CTTCACCTGCTCCACTGCCTTG
Tnf	GCCCAGACCCTCACACTCAGAT	AGCCTTGTCCCTTGAAGAGAACCT
Nfkb	GTCTTACACTTAGCCATCATCCACCTC	ATCCTCTACTACATCTTCCTGCTTGGT
Muc2 [52]	TGCTGCTGACGAGTGGTTGGTG	CGGACGCTTGGTGGTGAGGC
Muc4 [52]	GGCCCGCTTGGACATTTGGTGA	AGTCTCCCAGCCCGTTGAAGGT
Tjp3 [79]	TCGGCATAGCTGTCTCTGGA	GTTGGCTGTTTTGGTGCAGG
Ocln [32]	CCGGCCGCCAAGGTTC	GCTGATGTCACTGGTCACCTA
Cldn2 [52]	GTCATCGCCCATCAGAAGAT	ACTGTTGGACAGGGAACCAG
Cldn5 [80]	GCTCTCAGAGTCCGTTGACC	CTGCCCTTTCAGGTTAGCAG
Nrf2 [81]	CCTCCGCTGCCATCAGTCAGT	TCGGCTGGGACTCGTGTTCA
Cox2 [81]	GAAGTCTTTGGTCTGGTGCCTG	GTCTGCTGGTTTGGAATAGTTG

Supplementary Files

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