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Investigation of Inflammation Alleviation in an IBD Mouse Model Using the Butyrate-Producing Strain and Tributyrin

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Investigation of Inflammation Alleviation in an IBD Mouse Model Using the Butyrate-Producing Strain and Tributyrin

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ABSTRACT

Investigation of Inflammation Alleviation in an IBD Mouse Model Using the Butyrate-Producing Strain and Tributyrin

Inflammatory Bowel Disease (IBD) is a chronic condition characterized by intestinal inflammation and gut dysbiosis, with limited treatment options beyond immune-targeted therapies. This study investigates the therapeutic potential of BM107, a *Bacillus subtilis* strain, in combination with tributyrin (TB), a butyrate prodrug administered as a custom TB diet, in a DSS-induced mouse model of colitis. BM107, isolated from human fecal samples for its superior TB-degrading ability, was confirmed to be non-hemolytic and antibiotic-sensitive. The custom TB diet was developed to ensure consistent and effective delivery of TB, and the effects of BM107 and TB, both individually and in combination, were evaluated.

Co-administration of BM107 and the TB diet significantly improved clinical outcomes, including reduced Disease Activity Index (DAI) scores, increased colon length, and restored body weight. The TB 107 group also exhibited a restored gut microbiome composition, with increased abundance of beneficial bacteria such as *Akkermansia muciniphila* and *Mediterraneibacter butyricigenes*, and decreased levels of pro-inflammatory taxa like *Turicibacter bilis*. Butyrate levels in the cecum content of the TB 107 group reached levels comparable to the healthy control group, highlighting the efficacy of the treatment in restoring gut homeostasis.

These findings demonstrate that BM107, combined with the TB diet, represents a safe and effective therapeutic strategy for addressing gut dysbiosis and inflammation in IBD. This novel approach offers promise for the development of bacterial therapeutics to improve patient outcomes and support intestinal recovery.

Key words : tributyrin, butyrate, esterase, microbiome, IBD

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I. INTRODUCTION

1.1. IBD

Inflammatory Bowel Disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is chronic inflammation that occurs throughout the gastrointestinal (GI) tract (Xavier & Podolsky, 2007). In recent years, the prevalence of IBD has increased worldwide, not only among the elderly but also in younger populations. Common symptoms include persistent diarrhea, abdominal pain, rectal bleeding, bloody stools, weight loss, and fatigue.
The complexity of IBD arises from its multifactorial etiology, including genetic predisposition, environmental triggers, and dysregulation of the immune system. For example, a family history of IBD increases the likelihood of disease onset, and immune responses to environmental factors can exacerbate inflammation. Moreover, obesity and Western dietary habits have been linked to gut dysbiosis, ultimately contributing to intestinal barrier dysfunction. In the current treatment landscape for Inflammatory Bowel Disease (IBD), non-biological therapies play a pivotal role, with 5-aminosalicylic acid (5-ASA). This medication is widely used for its effectiveness in managing mild to moderate cases of IBD, particularly ulcerative colitis (UC) (Magro et al., 2020).

1.2. Short-chain fatty acids (SCFAs)

Short-chain fatty acids (SCFAs) are carboxylic acids with aliphatic tails of fewer than six carbons, primarily produced through the fermentation of indigestible polysaccharides by gut microbiota. The most prominent SCFAs in the intestine are acetate (C2), propionate (C3), and butyrate (C4). SCFAs play a pivotal role in maintaining gut homeostasis by supporting a balanced microbial community composed of bacteria, archaea, fungi, and bacteriophages

In the intestinal mucosa, SCFAs exert various beneficial effects on intestinal epithelial cells (Parada Venegas et al., 2019) including a proliferation of cells (Wong et al., 2006), ATP synthesis of colonocytes, the integrity of the barrier, and enhancing immunity (Salvi & Cowles, 2021).

SCFAs are used and taken up by colonocytes in the intestine, and they are transported using proton and monocarboxylate transporters (Ramos Meyers et al., 2022). That is some SCFA are absorbed by passive diffusion and some by solute transporters including the proton-coupled monocarboxylate-transporter 1 (MCT1/SLC16A1) and the sodium-coupled monocarboxylate-transporter 1 (SMCT1/SLC5A8) (Miyauchi et al., 2004; Ritzhaupt et al., 1998). Under 10 carbon fatty acids are diffused into the inner mitochondrial membrane in the epithelial cytoplasm. Their uptake ensures the availability of SCFAs for local energy metabolism and systemic signaling, reinforcing their critical role in intestinal health.

In addition, SCFAs' active signal cascades for immune modulation through G-protein coupled receptors (GPCRs) such as GPR41, GPR43, and GPR109A. In the human colon, the total SCFA concentration is low in the terminal ileum at 13± 6 mmol/kg, is high in the cecum at 131± 9 mmol/kg, and is 80 ± 11 mmol/kg in the descending colon (Cummings et al., 1987).

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1.3. Tributyrin and Butyrate

Butyrate, one of the most extensively studied SCFAs, is essential for colonic health. Approximately 70-90% of butyrate produced in the gut is consumed by colonocytes, where it serves as a primary energy source by fueling ATP production (Wong et al., 2006). Beyond its metabolic role, butyrate exerts additional therapeutic effects, particularly through its interaction with G-protein coupled receptors (GPCRs) such as GPR43 and GPR109A, expressed on colonic epithelial cells. Among these, GPR109A, encoded by the *Niacr1* gene, specifically responds to butyrate and niacin in colonic epithelial and immune cells (Singh et al., 2014). Activation of GPR109A enhances anti-inflammatory responses by promoting the activity of colonic macrophages and dendritic cells, further supporting intestinal health (Singh et al., 2014). Also, that can help the differentiation of Treg and IL-10-producing T cells. CD4+ T regulatory cell (Treg) exerts maintaining homeostasis in the colon, so Treg is abundant in colonic mucosa (Atarashi et al., 2011). Additionally, butyrate inhibits histone deacetylase (HDAC), thereby regulating colonocyte proliferation and reducing the risk of colon carcinogenesis (Donohoe et al., 2012; Hamer et al., 2008).

Despite its numerous benefits, direct supplementation with butyrate poses challenges, including its rapid metabolism, unpleasant odor, and gastrointestinal side effects. Tributyrin (TB), a triglyceride composed of three butyrate molecules esterified to a glycerol backbone, offers a solution by acting as a prodrug that releases butyrate gradually in the gut. Naturally present in foods like butter, TB has been shown to alleviate gut dysbiosis and repair intestinal damage in antibiotic-treated mice (Yang et al., 2023). Its safety, ease of consumption, and slow-release properties make TB a promising candidate for therapeutic applications in IBD and other intestinal disorders.

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II. MATERIALS AND METHODS

2.1. Isolation and maintenance of BM107

Dilute human feces from human fecal banks (YS Flora ®) and spread on TB agar plate (TSB, 2% Tributyrin). Then incubate the plate at 37°C for 24h, and isolate the colonies degrading TB by making a halo on the plate. To reconfirm Tributyrin degradation, the bacterial candidates were first grown in 2 mL of LB broth with a 2% inoculation of each strain stock (BM107 and other strains) at 37°C overnight. The following day, the cultures were adjusted to an OD of 1, and 10 µL of each culture was spotted onto TB agar plates (12.5 g LB broth, 10 g agar, 5 mL Tributyrin agar supplement). Plates were incubated at 37°C for 24 hours to observe degradation activity (Jung et al., 2021). Among TB-degrading bacteria select one strain. Furthermore, the initially isolated strain was stored in glycerol stock at -80°C for further studies. Antibiotic susceptibility test and hemolysis test were carried out following each European Food Safety Authority (EFSA) and Americal Society for Microbiology.

2.2. Esterase enzyme activity assay

Tributyrin esterase activity was evaluated using the chromogenic substrate p-nitrophenyl acetate (p-NPA, Sigma Aldrich, USA), following previously described methods (Quinn et al., 1982). The production of p-nitrophenol was measured at 410 nm using a spectrophotometer. For the assay, the isolated bacteria were cultured for 6 hours in LB broth, and broth cultured medium and the supernatant by filtration with 0.2 µm filter were used as enzymes for the assay.

2.3. Whole genome sequencing of BM107

HiFi reads generated from the PacBio SequelIIe system were assembled using the Microbial assembly application in SMRTlink 11.1.0.166339, based on the Hierarchical Genome Assembly Process (HGAP) 4. Default settings were used. Illumina raw reads were filtered for quality, retaining those with a phred score ≥30 for 90% of the bases, and adapters were trimmed using Trimmomatic 0.38. The assembly was corrected using Pilon v1.21 and the high-quality NovaSeq reads. Assembly quality was assessed using BUSCO. Prokka v1.14.6 was employed for gene prediction, while further annotation was conducted with InterProScan and psiblast. Circular contig maps were generated with Circos v0.69.6.

2.4. Growth curve

BM107 were inoculated into 10ml LB broth one day before and incubated at 37°C for 24h.The next day we transferred the bacteria to a new 20ml LB broth to make OD 0.05 at 600nm and repeated triple. Then, the OD was measured at 600nm per time.

2.5. Differences in the amount of butyrate by time points.

The method of growing bacteria was the same as above but additionally, 10mM TB was treated in the broth, and the difference was collecting cultured broth 1ml and cell down then filtered 0.2 µm Minisart® Syringe Filters (Sartorius, Germany) per time. The samples were kept at -20°C

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until a measurement, and butyrate was measured by high-performance liquid chromatography (HPLC) using Ultimate3000 (Thermo Dionex, USA) with a UV detector (210 nm) and refractive index detector (RefractoMAX520, Japan). For analysis, the Aminex 87H column (300 × 10 mm, Bio-Rad, CA, USA) was used and 0.01 NH2SO4 (Fluka, USA) were used as an eluent.HPLC analysis was performed by the Joint Institute of Agricultural and Life Sciences (NICEM, Seoul National University College of Agricultural and Life Sciences).

2.6. In vivo test

Bacteria culture conditions

BM107 and *Bacillus subtilis* KCTC3135^T were cultured aerobically in Luria-Bertani (LB) broth at 37°C for 20h with a shaking incubator at 210 rpm and suspended in sterilized 1x phosphate-buffered saline (PBS) for making 10^8 CFU/100ul. To confirm 10^8 CFU/100ul in all animal experiments, tested uniformed OD at 600nm and diluted cells and spread on LB plates.

Scheme/ DSS

We used 9(or 12)-week-old male C57BL/6 mice purchased from Orient Bio (Sungnam, Korea). The mice were provided with sterile water and food. All experiments involving mice were conducted following the guidelines of the Department of Animal Resources of the Yonsei Biomedical Research Institute and regulations within ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines. This study was approved by the Committee on the Ethics of Animal Experiments at Yonsei University College of Medicine (permit numbers, 2021-0139).

After an adaptation period of one week until 9(or 12)-week-old, the mice were administered 2% dextran sodium sulfate (DSS; molecular weight, 36,000–50,000 Da; MP Biomedicals, OH, USA) in their drinking water for 5 days, and replaced with 1% DSS for days, and then replaced with normal drinking water for the following 5 days. The mice of all groups were fed normal chow (NC, 13.12% of energy from fat, PicoLab Rodent Diet 20, LabDiet 5053, Texas) for 5 days and changed their diet to Tributyrin diet (TB) on the 6th day only in TB PBS, TB 107, and TB KCTC group, until last date of sacrifice.

There are six groups, the first group was fed NC, had no DSS treatment, and orally gavaged only PBS 100ul (CON PBS). Second NC and DSS were treated and orally gavaged PBS 100ul (NC PBS). Third NC and DSS were treated, and orally gavaged BM107 100ul (NC 107). Fourth was fed a TB diet, treated with DSS, and orally gavaged PBS 100ul (TB PBS). Fifth TB and DSS were treated, and orally gavaged BM107 100ul (TB 107). Sixth TB and DSS were treated, and orally gavaged *B. subtilis* KCTC3135^T 100ul (TB KCTC). Following each treatment like PBS or BM107 in the 6th,7th,8th, and 9th days, the mice are treated by oral gavage. All animal experiments were conducted with a sample size of at least n = 10 per group. Data for body weight and DAI scores included both mice that died due to DSS treatment and those that survived. However, experiments performed after sacrifice were conducted only on surviving mice.

TB diet

A customized TB diet was made by Glyceryl tributyrate (Sigma-Aldrich, 91010) 26.67mL in 1kg chow diet (5053).

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DAI score

Disease activity index (DAI) scores were taken following a previous study (Chen et al., 2007), but based on some modified criteria. This information is summarized in Table 1.

Colon length

When sacrificed the mice's colon was harvested from the cecum to the rectum and measured by a ruler.

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Table 1. Criteria of disease activity index(DAI) score

Score	Decrease in weight (%)	Stool consistency	Occult/gross rectal bleeding
0	0	Normal	Normal
1	1-6	Lightening or slight looseness	Occult blood +
2	6-11	Lightening and slight looseness	Occult blood++
3	11-18	Loose stools	Occult blood+++
4	>18	Diarrhea	Gross bleeding

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2.7. Histological analysis

The mice colons were cut into 1/3 pieces, harvested the most, opened longitudinal, and fixed in 3.7% formaldehyde. Tissues were paraffin-embedded and 5μm sections were stained with Hematoxylin-Eosin (H&E) and Alcian Blue-Periodic Acid-Schiff (AB-PAS). The slides of stained sections were analyzed using an optical microscope (BX53M; Olympus).

2.8. LC-MS

All steps were conducted under a 4°C condition. When sacrificing the mice, we prepped the cecum content and kept them on ice. Weighted the cecum 100mg and put 1ml of PBS per 100mg of cecum content. Centrifuge for 15 minutes at 8,000rpm after that, take the supernatant carefully and put 4 times the amount of methanol in the supernatant. Centrifuge for 15 minutes at 8,000rpm again, then take the supernatant carefully more than 300μl to a new tube. The samples were kept at -20°C until butyrate was measured by Liquid chromatography-mass spectrometry (LC-MS) using Vanquish Flex UHPLC system (Thermo Fisher Scientific Inc.). For the analysis, a Waters Cortecs C8 column (2.1 x 150 mm, 1.6 μm) was used and maintained at 50°C. The mobile phases consisted of A: 2 mM ammonium acetate and B: methanol (MeOH), with a flow rate of 0.25 mL/min. The Joint Institute of Agricultural and Life Sciences (NICEM, Seoul National University College of Agricultural and Life Sciences) performed LC-MS analysis.

2.9. Microbiome composition analysis

DNA was extracted from fecal samples using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The extracted DNA was quantified with a Quant-IT PicoGreen (Invitrogen). Sequencing libraries were prepared using the Illumina 16S Metagenomic Sequencing Library protocols to target the V3 and V4 regions. For PCR amplification, 2ng of genomic DNA was used in a reaction mixture containing 5X reaction buffer, 1 mM dNTP mix, 500 nM of each universal forward and reverse primer, and Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA). The thermal cycling conditions for the first PCR included an initial denaturation at 95°C for 3 minutes, followed by 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final extension at 72°C for 5 minutes. The universal primer sequences with Illumina adapter overhangs for the first amplification were: V3-F: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′, V4-R: 5′-GTC TCG TGG CTC GGA GAT GTG TAT AAG AGA CAG GAC TAC HVG GGT ATC TAA TCC-3′. The first PCR product was purified using AMPure beads (Agencourt Biosciences). For the final library construction, 2 μL of the purified first PCR product was subjected to a second round of PCR with NexteraXT Indexed Primers under the same conditions as the first PCR but with 10 cycles. The final PCR products were purified with AMPure beads. Quantification of the purified libraries was performed using qPCR as per the KAPA Library Quantification kits for Illumina Sequencing platforms, and quality assessment was done using TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Paired-end sequencing (2×300 bp) was conducted by Macrogen on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

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2.10. Statistical analysis

The data are presented as means ± standard deviation (SD). All data statistical analyses were conducted using GraphPad Prism version 9.4.1 (GraphPad Software Inc., La Jolla, CA, USA). Discrepancies between groups were assessed using a one-way analysis of variance (ANOVA) or Student's t-test, with p-values < 0.05 considered to be statistically significant. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.)

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III. RESULTS

3.1. Isolation of Tributyrin-degrading Strain

Tributyrin (TB) has three ester bonds linking for a glycerol and 3 butyrate. When bacteria possess esterase, they degrade tributyrin (TB) (Bornscheuer & Kazlauskas, 2006), forming a transparent halo zone. This was used as a selection method. Most strains exhibiting tributyrin degradation, evidenced by halo formation on TB agar plates, were identified as *Bacillus subtilis*. This species, which is included in the GRAS (Generally Recognized As Safe) list and is known for its high number of esterases (Soumya & Kochupurackal, 2020), was selected for further analysis(Fig. 1A). We also tested the hemolysis ability of the bacteria forming a halo and only two strains (BM107 and 23T122) didn't have hemolysin (Fig. 1A). Thus, BM107, which formed the larger halo, was selected among the two proven safe strains. For additional safety evaluation, the antibiotic resistance profile of this strain was assessed (Table 2). Results confirmed no resistance to vancomycin, gentamicin, kanamycin, erythromycin, clindamycin, tetracycline, and chloramphenicol. Resistance to streptomycin, however, was observed, which is an intrinsic characteristic of *Bacillus subtilis* (Adimpong et al., 2012). This trait has also been reported in commercially available *B. subtilis* probiotics, confirming that it does not pose a safety concern.

Next, we confirmed the enzyme activity of BM107. The strain was cultured in LB broth under aerobic conditions for 6 hours, and enzyme activity was assessed using the culture and the filtered supernatant. No significant changes were observed in either the control (LB broth) or the supernatant. However, in the culture containing the strain, 4-nitrophenyl acetate (p-NPA) was hydrolyzed to 4-nitrophenolate over time(Fig. 1B). This indicates that the isolated strain exhibits esterase activity.

Whole genome sequencing of BM107 was performed. The whole genome sequencing of the bacteria yielded 4,215,642bp with an average G+C content of 43.5%. RNA genes are 30 rRNA genes, 87 tRNA genes, and 1 tmRNA gene. The bacteria is predicted to contain 4,213 protein-coding genes among 4,331 total genes (Table 3). Among these, genes encoding esterase and lipase were identified (Table 4).

To confirm whether this strain increases butyrate production when TB is provided as a substrate and whether its growth is unaffected by TB, BM107 was inoculated under identical conditions with the only variable being the presence or absence of TB. Additionally, in the setup treated with 10 mM TB, the concentration of butyrate was measured over time to determine whether it increased. The growth curve analysis showed that BM107 grew well in LB broth, regardless of the presence of 10 mM tributyrin (TB) (Fig. 1C). When 10 mM TB was added, SCFA analysis using HPLC revealed a continuous increase in butyrate production over time (Fig. 1D). However, butyrate levels plateaued once the bacteria reached the stationary phase. This suggests that butyrate production is closely correlated with the growth rate of BM107, increasing proportionally during the exponential phase but stabilizing once growth ceased.

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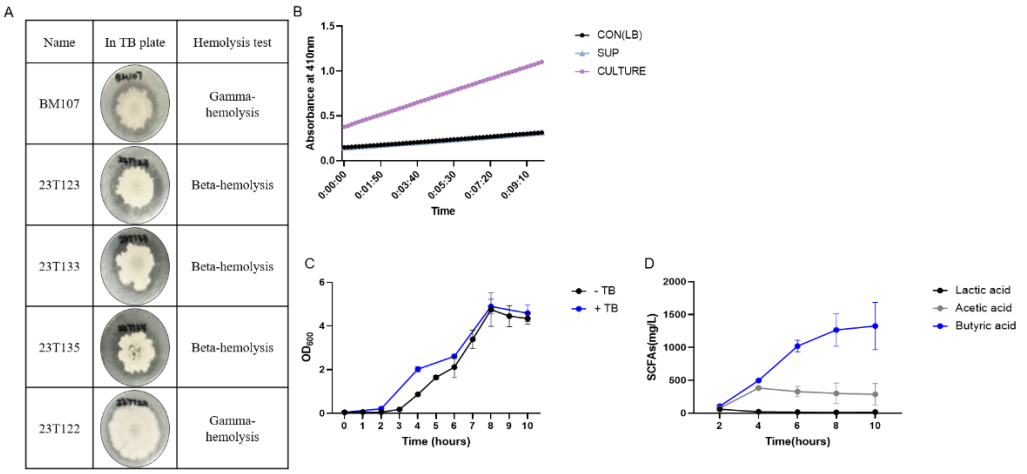

Figure 1. Selection of TB degrading strain from human feces (A) Bacillus subtilis strains were isolated from human fecal samples using TB agar plates. To ensure safety, hemolysin tests were conducted, and only non-hemolytic strain was chosen. (B) Esterase activity test of BM107 (C) Growth curve of BM107 with and without TB. As there is no significant difference between them, it can be seen that TB does not inhibit growth. (D) Changes in culture supernatant SCFAs during growth curve measurement. The concentration of butyrate gets higher time-dependently.

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Table 2. Antibiotics resistance

Antibiotics	Cut-off (mg/L)	MIC (mg/L)	Result
Vancomycin	4	0.5	S
Gentamicin	4	2	S
Kanamycin	8	6	S
Streptomycin	8	512	R
Erythromycin	4	0.125	S
Clindamycin	4	1.5	S
Tetracycline	8	3	S
Chloramphenicol	8	2	S

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Table 3. The genome feature of BM107

Feature	Value
Genome size (bp)	4,215,642
G + C content	43.5%
Total number of genes	4,331
Total size of protein-coding genes	4,213
Protein-coding genes	4.213
Average CDs size (bp)	876
rRNA number	30
tRNA number	87
tmRNA number	1

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Table 4. Predicted esterase in BM107

No.	Protein name
1	Putative esterase YxiM
2	Carboxylesterase
3	Putative esterase
4	Extracellular esterase EstB
5	Putative carboxylesterase nap
6	Carboxylesterase YbfK
7	Lipase EstA

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3.2. Superior Recovery Effects of BM107 Over the Type Strain in a DSS-Induced Colitis Model

Based on our previous experiments, we confirmed that BM107 can degrade tributyrin (TB) to produce butyrate. Butyrate is well-known for its ability to reduce intestinal inflammation and alleviate colonic injury (Venkatraman et al., 1999). Therefore, BM107, capable of producing butyrate from TB, was co-administered with TB in a DSS-induced mouse model to evaluate its effects on inflammation reduction and recovery. A customized TB diet was formulated to ensure efficient delivery, maintaining an intake of 60-80 μL. This diet was designed to provide a consistent intake and was administered in a form optimized for absorption and efficiency. To determine whether the recovery effect was due to the anti-inflammatory properties of *Bacillus subtilis* as a species or the specific functionality of BM107, we compared BM107 with the type strain *Bacillus subtilis* KCTC3135 in a DSS-induced colitis model.

Mice were divided into four groups with different treatments, as outlined in Fig. 2A. Colitis was strongly induced by administering 2% DSS for 5 days followed by 1% DSS for 4 days (Fig.2 A). This resulted in weight loss, bloody diarrhea, and watery stools, reflected in elevated Disease Activity Index (DAI) scores. In the TB KCTC group and TB 107 group, significant reductions in DAI scores (Fig. 2B) and improved survival rates (Fig. 2D) were observed on the final day (day 14) compared to the DSS-treated control group (NC PBS). However, with respect to colon length (Fig. 2C), the TB KCTC group did not show a significant difference compared to the NC PBS group. In contrast, the TB 107 group demonstrated significant recovery, with an increase in colon length compared to the NC PBS group. Additionally, H&E staining results (Fig. 2E) from the TB KCTC group revealed delayed recovery of intestinal cells and inflammation. In contrast, the TB 107 group displayed continuous intestinal cell morphology similar to that observed in the CON group, indicating more effective recovery. These results suggest that treating the DSS-induced model with TB and *Bacillus subtilis* demonstrates the recovery effects of the species itself; however, BM107 exhibited superior efficacy in promoting recovery.

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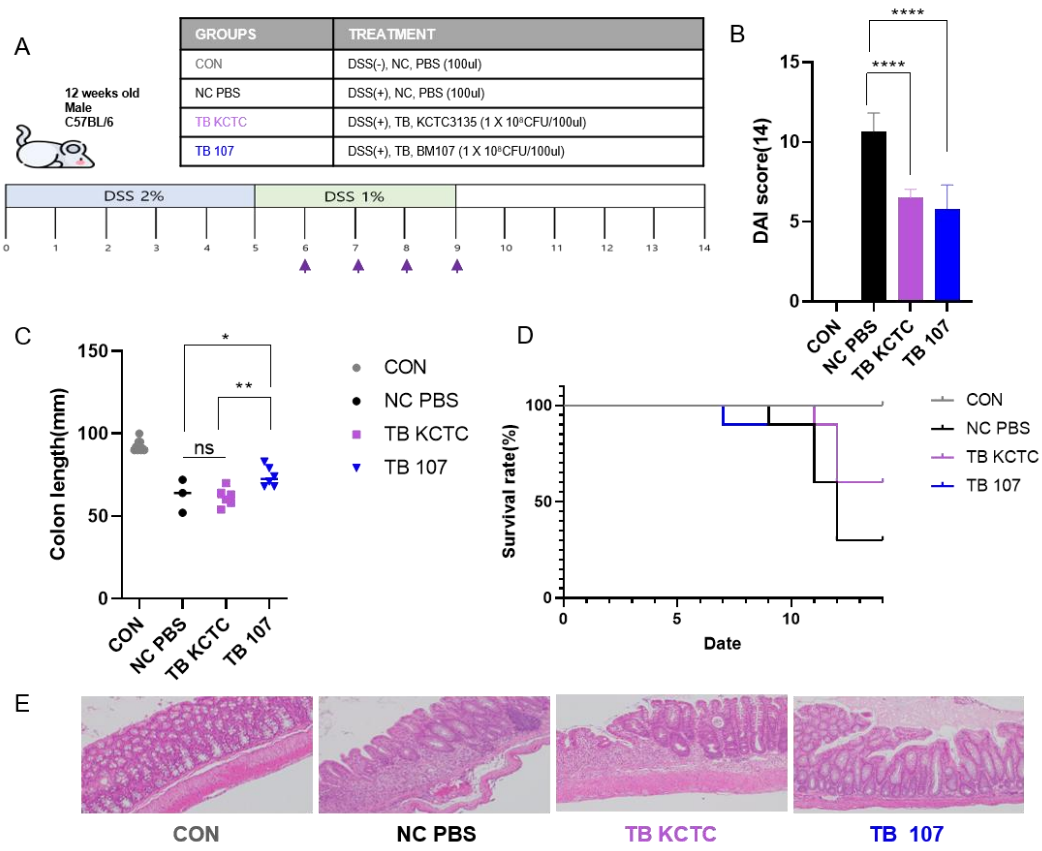

Figure 2. Compared with Bacillus subtilis KCTC3135^T and BM107
(A) Schematic diagram of DSS-induced colitis model, (B) disease activity index (DAI) score on the final day (day 14). (C) The colon length of the mice was measured after sacrifice. (D) The survival rate indicates the number of surviving mice in each group. (E) Representative images of colon tissues stained with hematoxylin and eosin (H&E). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$ compared to each group using One-way ANOVA.

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3.3. Effect of BM107 and Tributyrin on Disease Activity in DSS-Induced Colitis

To further investigate the specific functionality of BM107, which combines the general benefits of *Bacillus subtilis* with its enhanced recovery capability, additional experiments were conducted using the DSS-induced model. The study was divided into five groups, as outlined in Fig. 3A.

In the TB 107 group, recovery from weight loss began on day 10, as indicated by the percentage change from initial body weight (Fig. 3C). Similarly, the DAI score graph (Fig. 3B) shows significant improvement in the TB 107 group by day 14 compared to the NC PBS group. These results suggest that the combination treatment with BM107 and TB promoted recovery, even under severe conditions. On day 14, after sacrificing the mice, colon length was measured. The TB 107 group demonstrated the most significant improvement in colon length compared to all other groups (Fig. 3D). In contrast, neither the NC 107 group, treated with only the strain, nor the TB PBS group, treated with only TB, showed significant differences in DAI scores or colon length compared to the NC PBS group However, recovery was observed in the NC 107 group, as indicated by the percentage change from initial body weight (Fig. 3C).

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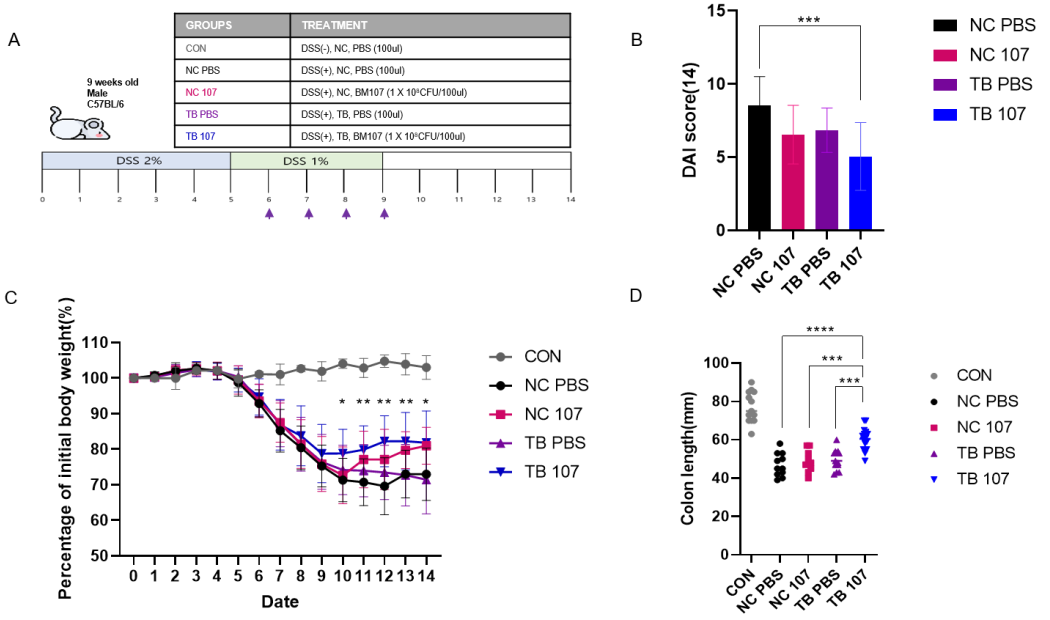

Figure 3. The effects of BM107 with TB in the DSS model (A) Schematic diagram of DSS-induced colitis model, (B) disease activity index(DAI) score on the final day (day 14). (C) Percentage of initial body weight (%) and (D) The colon length of the mice was measured after sacrifice. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$ compared to each group using One-way ANOVA.

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3.4. Synergistic Effects of TB and BM107 on Intestinal Recovery and Butyrate Restoration in a DSS-Induced Mouse Model

On day 14, after sacrificing the mice, the colon morphology of the TB 107 group resembled that of the healthy CON group, with well-formed feces observed (Fig. 4B). Additionally, the morphology of the cecum and intestinal moisture levels in the TB 107 group were restored to levels comparable to the CON group, in contrast to the NC PBS group. Examination of intestinal tissues further revealed significant improvement in the TB 107 group, similar to the CON group (Fig. 3A).

Hyper-inflammatory responses, characterized by neutrophil infiltration, were prominent in the NC PBS group but were markedly reduced in the TB 107 group, as evidenced by H&E staining. Furthermore, AB-PAS staining revealed substantial recovery of goblet cells, which are essential for maintaining intestinal barrier function (Vidal-Lletjós et al., 2019), with the TB 107 group showing the most significant restoration.

Interestingly, the NC 107 and TB PBS groups, which showed less pronounced effects in earlier analyses, also demonstrated tissue recovery in staining results. This suggests that both BM107 and TB alone contributed to tissue recovery, though the fastest and most comprehensive recovery was observed when TB and BM107 were co-administered, particularly after the final oral gavage on day 9.

We hypothesized that co-administration of TB and the TB-degrading strain BM107 would generate butyrate and promote intestinal recovery in the DSS-induced mouse model. To evaluate this, we measured butyrate concentrations using LC-MS to determine whether they reached levels comparable to the CON group. The results revealed that the TB 107 group achieved butyrate concentrations similar to the CON group (Fig. 4C). Although the groups treated with BM107 or TB alone had slightly lower butyrate levels than the TB 107 group, their recovery was still evident.

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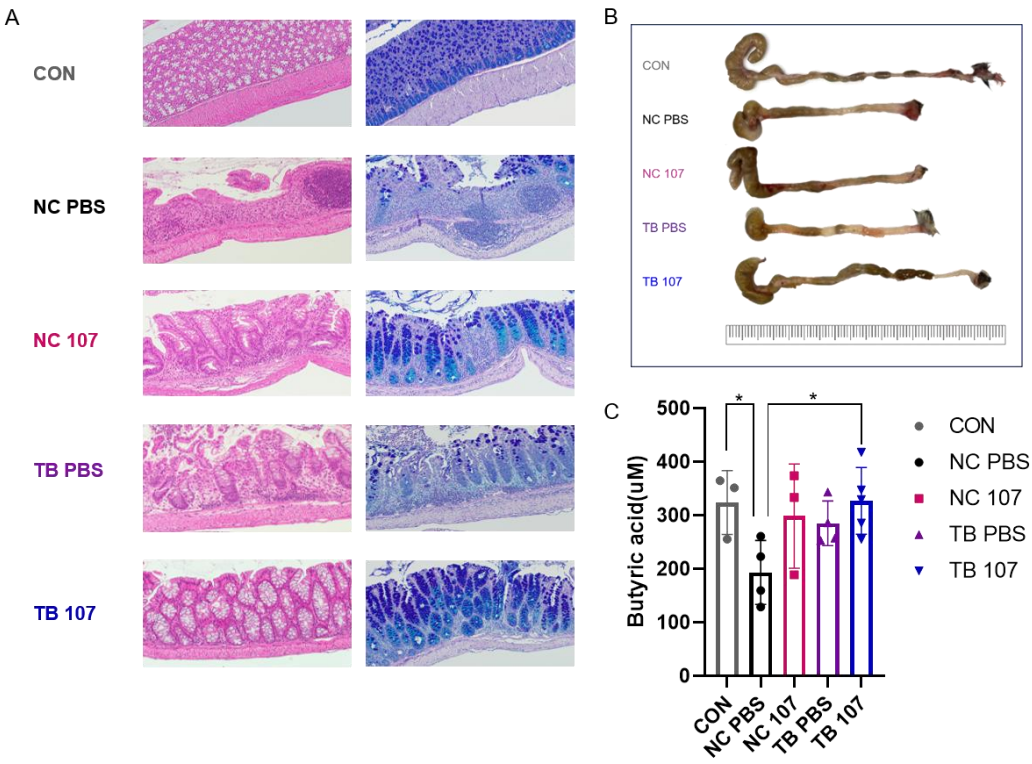

Figure 4. Histological, morphological, and biochemical assessment of BM107 and TB effects in a DSS-induced colitis model (A) Representative images of colon tissues stained with hematoxylin and eosin (H&E) and Alcian blue-PAS staining (AB-PAS) from indicated groups. (B) Representative image of mice colon and (C) Measurement of butyric acid concentration from mice cecum contents using LC-MS. *, $P < 0.05$ compared to each group using T-test.

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3.5. BM107 with TB impacts the gut microbiome

Dysbiosis, a key feature of IBD, can also serve as an indicator of recovery (Xavier & Podolsky, 2007). To evaluate the effects of the TB diet and BM107 treatment on the gut microbiome under colitis-induced conditions, we analyzed the cecum microbiome compositions of the NC PBS group and the TB 107 group, comparing them to the CON group.

The relative abundance of the gut microbiome for each group at the genus level is presented in Fig. 5A, *Phocaeicola* (26.1%, 28.74%, 24.8%), *Akkermansia* (12.7%, 9.7%, 25%), *Lactobacillus* (5%, 3.5%, 4.7%), *Turicibacter* (0%, 11.8%, 0.3%), *Limosilactobacillus* (2.5%, 1.8%, 4.1%), *Mediterraneibacter* (2.7%, 0.7%, 3.5%), *Romboutsia* (0%, 1.3%, 0.4%) were restored in the TB 107 group, similar to the patterns observed in the CON group (Fig. 5B). (Percentages are listed in order of CON, NC PBS, and TB 107 groups. In the TB 107 group, there was an increased abundance of *Limosilactobacillus reuteri*, *Lactobacillus intestinalis*, and *Mediterraneibacter butyricigenes*. *Mediterraneibacter butyricigenes*, a known butyrate producer from glucose, likely contributed to the restoration of butyrate levels in the TB 107 group. Prominent differences at both the species and genus levels were observed for *Akkermansia muciniphila* and *Turicibacter bilis*. In the TB 107 group, the proportion of *A. muciniphila* increased significantly, reflecting recovery similar to the CON group and even exceeding the levels observed in the CON group. Conversely, *T. bilis* was nearly undetectable in the non-inflammatory and recovered groups but constituted a substantial proportion in the NC PBS group, where inflammation persisted (Fig. 5C). Beta diversity, a measure of intestinal dysbiosis, revealed that the TB 107 group exhibited significant recovery compared to the NC PBS group (Fig. 5D).

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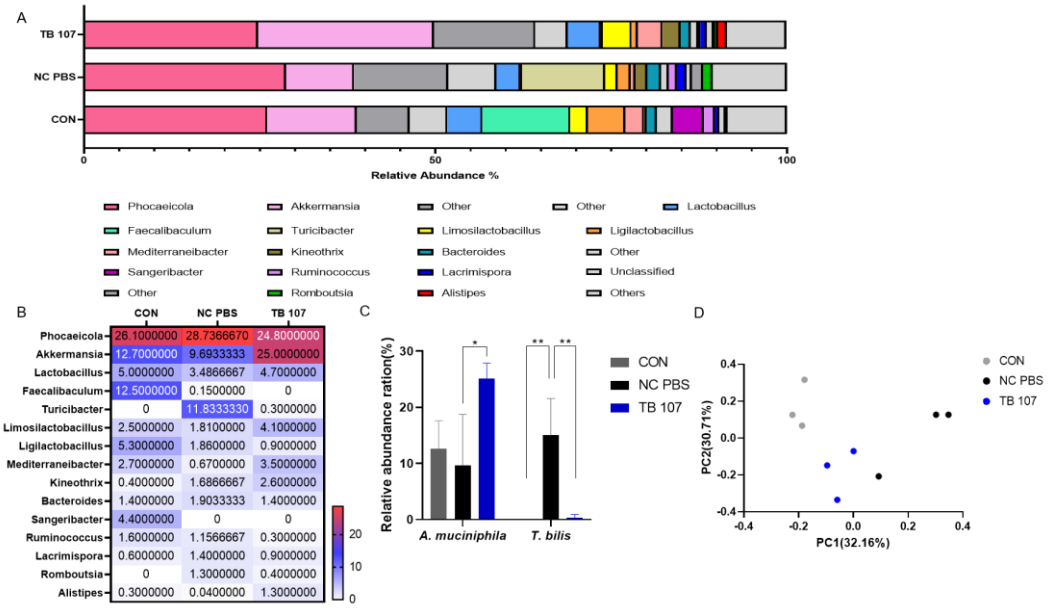

Figure 5. Alteration of the gut microbiome composition Changes occurred in the cecum contents of each group in the DSS-induced model, and the composition of the microbiome was examined at both the genus and species levels among CON, NC PBS, and TB 107 groups. (A) The microbiome composition at the genus level is depicted using bar charts. (B) The major microbes are presented. A heatmap created using GraphPad Prism illustrates the relative abundance of the genus, with the intensity of red or white indicating higher or lower abundance, respectively, in each group. (C) Species level of *Akkermansia* and *Turicibacter*. (D) Beta diversity to visualize the differences between CON, NC PBS, and TB 107 group.

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IV. DISCUSSION

With the increasing prevalence of IBD worldwide, treatment strategies should explore not only chemical medicines but also bacterial therapies involving TB. In addition to physical pain, IBD patients often experience anxiety and depression (Regueiro et al., 2017), so we have to consider their more comfortable and habitual treatments. Moreover, stress can exacerbate gastrointestinal pain and inflammation (Moloney et al., 2016). Currently, apart from targeted immune therapies, there is no definitive treatment for IBD, prompting this study to propose an alternative approach using TB-degrading bacteria. The bacteria were isolated from human fecal banks (YS Flora ®).

This strain, *Bacillus subtilis*, was selected for its proven safety profile and well-documented functionalities, including its ability to prevent gastrointestinal disorders, exhibit anti-inflammatory and immunomodulatory effects, and support anti-tumor activities (Sorokulova, 2013). As a spore-forming bacterium, *Bacillus subtilis* promotes the proliferation of beneficial lactic acid bacteria in human and animal intestines, reinforcing its established role as a probiotic (Hoa et al., 2000). Among the isolated *Bacillus subtilis* strains, the one with the highest tributyrin (TB) decomposition ability and established safety was designated BM107 (Fig. 1). This strain demonstrated superior recovery effects in the IBD model, highlighting its potential as a new therapeutic option. With its long history of use and well-documented safety profile (Hong et al., 2005), BM107 emerges as a promising candidate for mitigating intestinal inflammation and enhancing gut health.

In previous studies, we confirmed that direct administration of tributyrin (TB) had a positive effect in a DSS-induced mouse model, and similar results were observed in a strain of *E. coli* harboring an esterase gene (Jung et al., 2021). Based on this, we aimed to use a human-derived strain with esterase activity (Fig. 1) and designed a model with prolonged inflammation by administering DSS for 9 days. To minimize stress on the mice and simulate the natural intake of TB in humans, a once-daily oral gavage was planned, requiring an alternative TB delivery method. We developed a custom diet, allowing the mice to ingest 60-80μL of TB per day.

To demonstrate functionality in the animal model, the selected strain was compared with the type strain to evaluate both the general benefits of the *Bacillus subtilis* species (*B. subtilis* KCTC3135^T) and its specific ability to efficiently degrade and utilize TB. The type strain, which is well-studied and widely used as a probiotic (Garvey et al., 2023; Wang et al., 2019), showed similar growth conditions to BM107 and also formed halos on TB agar plates. Additionally, both strains possessed genes associated with esterase activity, as shown in Table 4. Both the TB KCTC group and the TB 107 group exhibited inflammation reduction. However, within the same time frame, the TB 107 group demonstrated superior recovery, as evidenced by increased colon length (Fig. 2C) and improved tissue staining images (Fig. 2E). This indicates that BM107's efficient TB degradation and utilization of the resulting butyrate played a pivotal role in accelerating disease recovery. These findings indicate that BM107 not only exhibits the general advantages of the *Bacillus subtilis* species but also possesses unique functionality that enhances its anti-inflammatory effects through the efficient degradation of TB.

To further explore the effects of co-administration versus individual treatments of TB and BM107, we designed an additional set of experiments with separate treatment groups. Unlike the type strain experiment set, which was conducted in 12-week-old mice, this study utilized 9-week-old mice. This younger age was selected to better mimic early-stage disease progression and susceptibility, providing a more comprehensive understanding of the therapeutic potential of TB

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and BM107. This age adjustment allows us to assess whether the treatment outcomes differ across developmental stages, particularly in the context of inflammation recovery and gut microbiota modulation.

In this prolonged DSS-induced IBD model, co-administration of BM107 and the TB diet showed a recovery effect, superior to the groups treated with either BM107 or TB alone, suggesting that the simultaneous delivery of both contributed to enhanced recovery (Fig. 3). As shown in Fig. 4A and Fig. 4C, recovery was observed in both the NC 107 group, treated with the strain alone, and the TB PBS group, treated with the TB diet alone. This indicates that the recovery functionality of the strain itself and the butyrate production through TB functioned effectively (Clarke et al., 2001; Wächtershäuser & Stein, 2000).

The gut barrier function is maintained through intestinal homeostasis. It can be regulated by the gut microbiota, an essential factor contributing to intestinal health that may be strengthened or weakened through interactions with the host (Sommer & Bäckhed, 2013). In particular, the degree of inflammation induced in the DSS model varies depending on the presence or absence of the gut microbiota, indicating that this model is highly influenced by the gut microbiota (Maslowski et al., 2009). Therefore, the altered gut microbiome composition in this model is of significant interest. *A. muciniphila* is the sole species within the Verrucomicrobia phylum was first isolated from the stool of a healthy individual by Derrien et al in 2004 (Derrien et al., 2004), and is known for reducing intestinal inflammation and strengthening the gut barrier (Earley et al., 2019). In this study, its abundance increased significantly in the TB 107 group, surpassing even the levels observed in the healthy control group (Fig. 5C). This indicates that the co-administration of TB and BM107 not only restores gut homeostasis but also created a more favorable intestinal environment, leading to an optimal niche for *A. muciniphila*. This indicates the potential of TB and BM107 to enhance gut health beyond baseline conditions. At both genus and species levels, *Turicibacter*, particularly *T. bilis*, was significantly increased in the NC PBS group, consistent with its reported dominance under colitis-induced conditions (Shang et al., 2021). Its persistence in dysbiotic, inflammatory environments contributes to the exacerbation of IBD pathology (Fig. 5B and 5C). In healthy intestinal conditions, *Lactobacillus* populations increase, contributing to elevated butyrate levels. In contrast, under colitis-induced conditions, *Turicibacter* and *Romboutsia* become dominant (Lee et al., 2019). This aligns with the results shown in Fig. 5B and 5C, highlighting the significance of the novel approach of co-administering the TB diet and BM107 in the IBD model.

The butyrate concentration in the cecum content of the TB 107 group increased to levels comparable to those of the healthy control (CON) group, indicating substantial recovery. In contrast, the NC PBS group, which did not recover sufficiently, showed lower butyrate levels. Notably, butyrate levels across all groups displayed minimal fluctuations, which can be attributed to the compound's role in maintaining homeostasis and its inherently short half-life (Egorin et al., 1999). Despite this limited variability, the TB 107 group achieved significant increases in butyrate concentration, highlighting the effectiveness of the treatment. These findings suggest that the combined administration of BM107 and TB could serve as a promising therapeutic option for IBD patients, offering a novel approach to restoring gut health and maintaining intestinal balance.

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V. CONCLUSION

This study highlights the potential of BM107, a *Bacillus subtilis* strain, as a novel therapeutic candidate for managing IBD. BM107 demonstrated superior tributyrin (TB) degradation capabilities, resulting in enhanced butyrate production, a critical factor in restoring gut homeostasis and reducing intestinal inflammation. The co-administration of BM107 and TB in a DSS-induced mouse model not only improved clinical outcomes, such as DAI scores, body weight recovery, and colon length but also significantly modulated the gut microbiota composition. Notably, beneficial bacteria such as *Akkermansia muciniphila* and *Mediterraneibacter butyricigenes* increased, while pro-inflammatory taxa like *Turicibacter bilis* decreased, further supporting the therapeutic efficacy of this approach. The results suggest that the combined use of BM107 and TB offers a promising, safe, and effective strategy for addressing gut dysbiosis and inflammation in IBD. This work provides a foundation for further exploration into bacterial therapeutics in IBD management, with potential implications for clinical translation.

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Abstract in Korean

염증성 장질환(IBD) 모델에서 Butyrate-Producing Strain과 Tributyrin을 활용한 항염증 효과 연구

염증성 장질환(IBD)은 장내 염증과 장내 미생물군 불균형을 특징으로 하는 만성 질환으로, 현재 면역 치료 외에는 제한적인 치료 옵션만이 존재합니다. 본 연구는 DSS로 유도된 대장염 마우스 모델에서 *Bacillus subtilis* 균주인 BM107과 뷰티레이트 전구체인 트리뷰티린(TB)을 병용한 치료 가능성을 평가하였습니다. TB는 제작한 TB 식이를 통해 투여되었으며, BM107은 TB 분해 능력이 우수한 균주로서 인간 분변 샘플에서 분리되었고, 비용혈성 및 항생제 감수성을 확인하였습니다. TB의 일관되고 효과적인 전달을 보장하기 위해 맞춤형 TB 식이가 개발되었으며, BM107 및 TB의 단독 및 병용 효과를 평가하였습니다.

BM107과 TB 식이를 병용 투여한 TB 107 그룹에서는 질병활성지수(DAI) 감소, 대장 길이 증가, 체중 회복 등 임상적 결과가 유의미하게 개선되었습니다. 또한, TB 107 그룹은 장내 미생물군 조성이 회복되었으며, *Akkermansia muciniphila*와 *Mediterraneibacter butyricigenes*와 같은 유익균의 증가와 함께 *Turicibacter bilis*와 같은 염증성 균주의 감소를 보였습니다. 더불어, TB 107 그룹의 맹장 내 뷰티레이트 농도는 건강한 대조군과 유사한 수준으로 회복되어 장내 항상성 복원에 효과적임을 입증하였습니다.

이러한 연구 결과는 BM107과 TB 식이를 병용한 치료가 IBD에서 장내 분균형 및 염증을 해결하기 위한 안전하고 효과적인 치료 전략임을 제시합니다. 본 연구는 장내 회복을 지원하고 환자 결과를 개선할 수 있는 박테리아 기반 치료제 개발의 가능성을 보여줍니다.

핵심되는 말 : 트리뷰티린, 낙산, 에스터레이스, 공생 미생물, 염증성 장 질환