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RESEARCH PAPER

Impact of short-chain fatty acid supplementation on gut inflammation and microbiota composition in a murine colitis model

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Abstract

Short-chain fatty acids (SCFAs) play a pivotal role in maintaining intestinal homeostasis. We aimed to investigate the effects of SCFA supplementation on gut inflammation and microbiota composition in a murine colitis model. Mice were fed with sodium butyrate or a mixture of SCFAs in the drinking water for 2 weeks, followed by 2% dextran sulfate sodium (DSS) for 7 d. After euthanasia, mouse colons were extracted to examine histological findings. Flow cytometry of the mouse colon tissues was performed to assess T cell differentiation. Changes in gut microbiota were assessed by high-throughput sequencing of the mouse feces. There were no significant differences in weight change, colonic length, or histologic inflammation score between the DSS, butyrate, and SCFA mix groups. However, flow cytometry revealed that both the expression of CD4+Foxp3+ regulatory T cells and of IL-17-producing T cells were increased in the butyrate and SCFA mix groups. Microbial compositions of the butyrate and SCFA mix groups were significantly different from those of the control and DSS groups in principal coordinate analysis. Relative abundances of the phyla Verrucomicrobia and Proteobacteria, species *Akkermansia muciniphila* and *Escherichia fergusonii* were increased in the butyrate and SCFA mix groups. Genera *Roseburia* and *Lactobacillus* showed a negative correlation with the degree of colitis, whereas genera *Escherichia* and *Mucispirillum* showed a positive correlation. SCFA supplementation did not result in a significant reduction in colon inflammation, but it promoted both regulatory T cell and IL-17-producing T cell expression, and increased both protective and aggressive gut microbiota.

Keywords: Short-chain fatty acids; Butyrates; Inflammatory bowel disease; Colitis; Gut microbiota.

1. Introduction

Inflammatory bowel disease (IBD), consisting of Crohn's disease and ulcerative colitis, is characterized by chronic recurrent inflammation of the gastrointestinal tract. IBD not only causes debilitating gastrointestinal symptoms, but also causes progressive bowel damage and complications including stricture, fistula, and abscess [1]. IBD has become a worldwide disease with rising incidence in newly developed countries [2,3]. Genetic susceptibility, environmental factors, gut microbiota, and immune response are involved in the pathogenesis of IBD [4].

Short-chain fatty acids (SCFAs), including butyrate, acetate, and propionate, are produced by fermentation of non-digestible carbohydrates by gut microbiota in the gastrointestinal tract, and play a crucial role in maintaining intestinal barrier integrity and homeostasis [5]. Butyrate, one of the main four-carbon SCFAs, serves as a major energy source for colonocytes and has been shown to induce an anti-inflammatory response by inhibiting the nuclear factor-κB pathway and reducing proinflammatory gene expression [6]. Decreased colonic SCFAs concentrations have been associated with IBD, diversion colitis, and antibiotic-associated diarrhea [7–9]. In addition, the reduction of SCFAs in patients with ulcerative colitis has been associated with disease activity, and patients in remission have higher levels of butyrate than those with active disease [10].

SCFAs have long been studied as a therapeutic agent for IBD, but studies have shown inconsistent results. Topical therapy with SCFAs has been shown to be effective in reducing clinical symptoms in patients with ulcerative colitis [11,12]. In contrast, other studies have failed to show significant benefits of SCFA treatment over placebo in patients with ulcerative colitis [13,14]. In Crohn's

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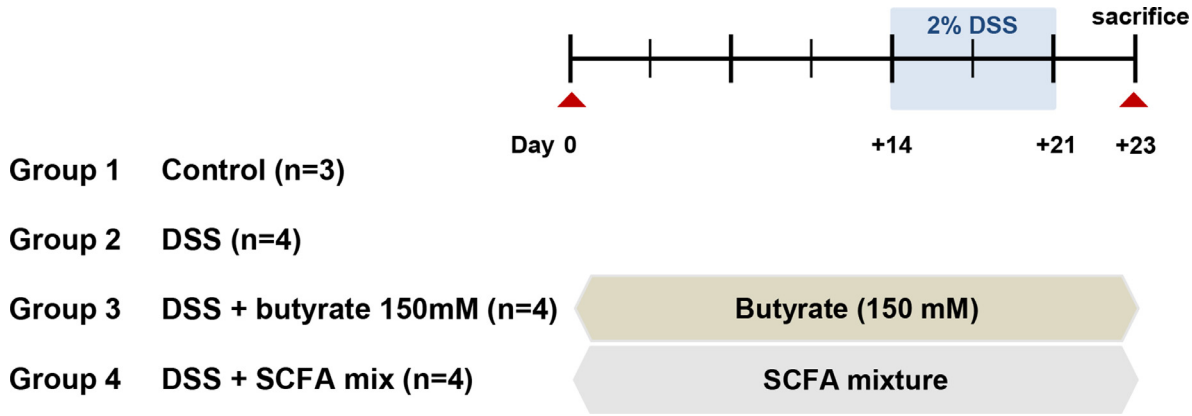

Fig. 1. Study protocol.
DSS, dextran sodium sulfate; SCFA, short-chain fatty acid.

disease, one uncontrolled pilot study has reported that butyrate administration was effective in inducing clinical improvement, but no randomized controlled trials have been conducted [15]. Moreover, most human studies have investigated the topical effect of butyrate, and little is known about the impact of SCFA supplementation on changes in gut microbiota. We therefore aimed to investigate the effects of oral administration of SCFAs on gut inflammation and microbiota composition in the dextran sulfate sodium (DSS)-induced murine colitis model.

2. Materials and methods

2.1. Animals

C57BL/6 mice (female, 6-weeks-old) obtained from Orient Bio (Seongnam, Korea) were used. Mice were co-housed in groups at 23±3°C, 50±20% humidity, in a 12/12-h light/dark cycle, with free access to food and water under specific pathogen-free conditions in an accredited animal facility at Hanyang University. Standard mouse chow (LabDiet 5053, Orient Bio, Korea) was supplied to all mice. To minimize animal suffering and determine humane endpoints, mice were monitored daily for signs of distress including weight change, hair loss, abnormal eye opening, reduced physical activity, and abnormal posture. The criteria for determining the humane endpoints are shown in Table S1. All experimental procedures were performed according to the guidelines outlined and approved by the Animal Experimental Ethics Committee of Hanyang University (approval number: HY-IACUC-20-0025).

2.2. Induction of colitis and administration of SCFAs

Following oral administration of 150 mM sodium butyrate or a mixture of SCFAs (67.5 mM acetate, 40 mM butyrate, 25.9 mM propionate) in the drinking water for 2 weeks, colitis was induced by feeding mice with water containing 2.0% DSS for 7 d. The control group was given normal drinking water without DSS. Subsequently, mice were divided into four groups: (1) control group (n=3), no DSS and no SCFAs; (2) DSS group (n=4), DSS without SCFAs; (3) butyrate group (n=4), DSS with sodium butyrate; (4) SCFA mix group (n=4), DSS with mixture of SCFAs (acetate, butyrate, propionate). Mice were euthanized 2 d after the last dose of DSS administration. No mice were found dead or met the humane endpoint before the end of the experiment. The study protocol is shown in Figure 1.

2.3. Gross and histological assessment

After euthanasia, the mouse colons were extracted to assess colon inflammation. Digital photographs were taken and the length of the colon was measured. Colon tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin for histological evaluation. Three of the authors who were blinded to the slide information measured histological scores by summing the following scores as described in a previous study: degree of inflammation (0–3), extent (0–3), crypt damage (0–4), and percent involvement (1–4) (Table S2) [16].

2.4. Fluorescence-activated cell sorter (FACS) analysis

Cell suspensions were prepared from the colonic lamina propria with reference to previous reports [17]. Intestinal fragments were cut open and washed three times with PBS to isolate mononuclear cells from the intestinal lamina propria. Washed gut pieces were subsequently cut into pieces 1–2 cm in length and incubated for 30 min at 37°C in PBS containing 5% fetal bovine serum, 500 mM EDTA, 1 M HEPES, 100 mM sodium pyruvate, and 1X penicillin/streptomycin. Tissue pieces were washed three times by vigorous shaking with warm PBS and incubated for 30 min with fresh medium containing collagenase D (1 mg/ml; Roche, Basel, Switzerland) and DNase I (1 mg/ml; Roche, Basel, Switzerland). The remaining intestinal pieces were filtered through 100-μm mesh, and the cell suspensions were spun by centrifugation at 431 x g for 8 min. After discarding the supernatant, the cell pellet was resuspended in 75% (wt/vol) Percoll (GE Healthcare, Buckinghamshire, UK), then 40% (wt/vol) Percoll was added to the suspension. After spinning by centrifugation at 1,350 g for 20 min, mononuclear cells were collected from the 75%/40% interphase. Cells were first preincubated with the monoclonal antibody 2.4G2 (anti-mouse CD16/CD32 mAb; BD Pharmingen, BD Biosciences, CA, USA) to block Fcγ receptors, after which the cells were washed and incubated for 40 min with the appropriate monoclonal antibody conjugates. Incubations were performed in a total volume of 100 μl PBS containing 2 mM EDTA and 2% (vol/vol) bovine serum. Cells were analyzed on a FACSCanto II instrument (BD Biosciences, CA, USA) with FlowJo software (TreeStar, BD Life Sciences, NJ, USA). The following antibodies were used for flow cytometry: anti-FoxP3 antibodies conjugated to FITC, anti-IFNγ antibodies conjugated to APC, anti-IL17A antibodies conjugated to PE, anti-IL10 antibodies conjugated to APC-Cy7, and anti-CD4 antibodies conjugated to PerCP-Cy5.5 (eBioscience, CA, USA).

2.5. DNA extraction and 16S rRNA gene sequencing

Before the mice were sacrificed, mouse feces were collected and stored immediately at -80°C. Stool DNA was extracted using PowerFecal DNA Isolation Kit (MOBIO Laboratories, CA, USA) according to the manufacturer's protocol. Briefly, 100 mg of stool was added to the dry bead tube containing 800 μl of bead solution and gently vortexed. Then, samples were homogenized using Precellys 24 homogenizer (Bertin Technologies, France) at 3,000 rpm for 30 s, paused for 30 s, and then homogenized again at 3,000 rpm for 30 s. Samples were centrifuged at 15,000 x g for 1 min, the supernatant transferred to the collection tube, and the remainder of the procedure was followed as recommended by the manufacturer.

DNA concentration was determined via 260/280 and 260/230 absorbance ratios measured on the Biospec-nano spectrophotometer (Life Science, MD, USA). To amplify the extracted DNA, primers for the V3-V4 region of the 16S rRNA gene were used as follows: forward, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. Gene amplification conditions were initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 40 s, primer annealing at 57°C for 40 s, and extension at 72°C for 60 s, with a final elongation at 72°C for 60 s. Amplified 16S rRNA PCR products were normalized and pooled using the PicoGreen, and the size of libraries were verified using the LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, MA, USA). Libraries were sequenced on the Illumina MiSeq platform (Illumina, CA, USA) and the sequence data were processed using QIIME version 1.8.0 [18]. The short or extra-long reads in the sequences were trimmed, and the filtered sequence was classified using CD-HIT-DUP. Chimeric reads were identified, and small noise sequences were removed. Sequences with 97% similarity with the NCBI 16S rRNA database among the remaining representative readings were classified as operational taxonomic units (OTUs). A taxonomy for each OTU representative sequence was assigned based on the NCBI taxonomy database, using the QIIME pipeline. The complete genome se-

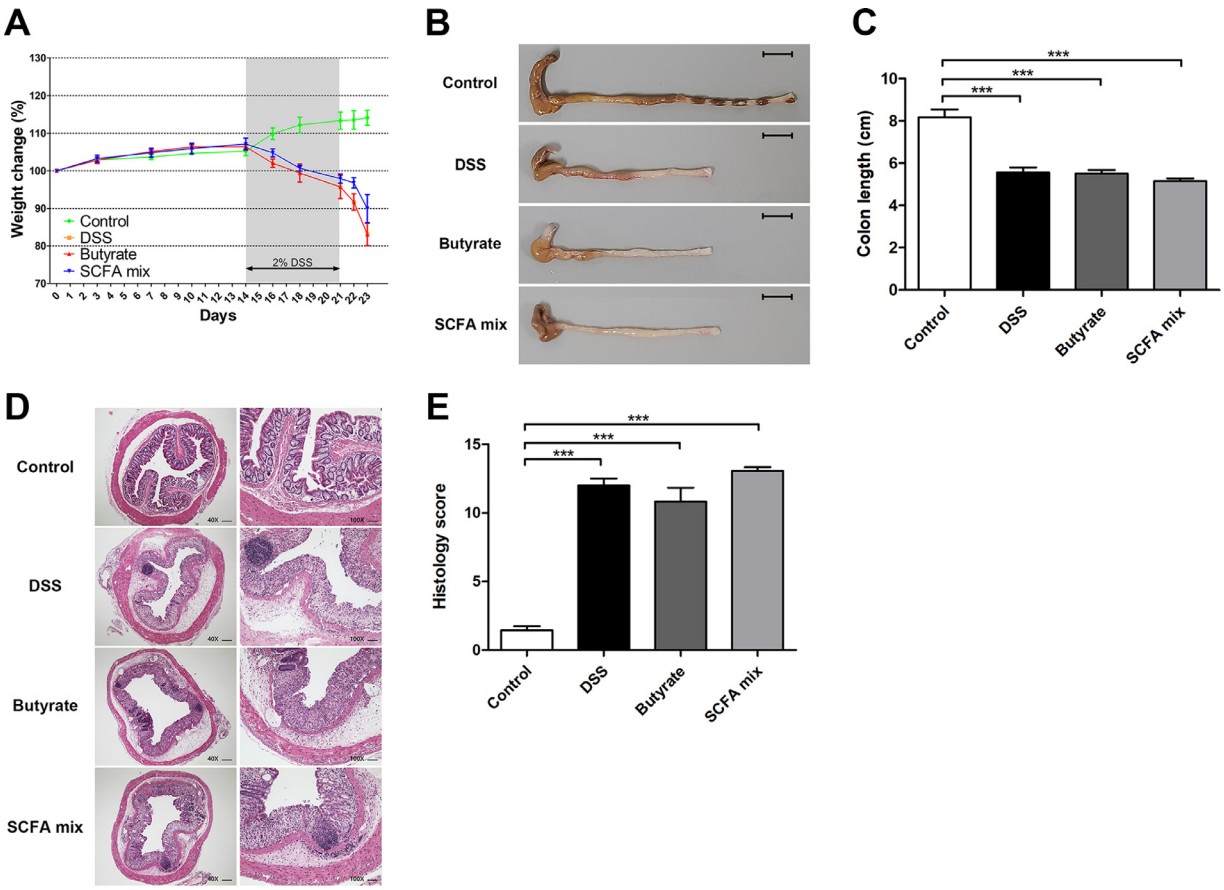

Fig. 2. DSS-induced colitis was not attenuated by oral administration of SCFAs.
(A) Weight change during the study period. Body weight loss was observed not only in the DSS-treated mice group, but also in the butyrate and SCFA mix groups.
(B) Representative images of extracted colons. Scale bar, 1 cm.
(C) Colonic length was significantly decreased in the DSS group compared to the control group (mean colon length: 5.55 cm *vs.* 8.17 cm, $P<.001$). However, there was no significant difference in colon length between the DSS, butyrate, and SCFA mix groups (mean colon length: DSS group, 5.55 cm; butyrate group, 5.50 cm; SCFA mix group, 5.15 cm).
(D) Representative histological findings. Images were taken at 40x and 100x magnifications. Scale bar, 200 μm.
(E) Histologic score of the mouse colon tissue (range from 1 to 14, higher scores indicate more severe colonic inflammation) was significantly increased in the DSS group than in the control. However, there were no significant differences in histologic scores between the DSS, butyrate and SCFA mix groups (mean histologic score: DSS group, 12.0; butyrate group, 10.83; SCFA mix group, 13.08).
Data were shown as mean with standard errors. Data for graphs were calculated using one-way ANOVA with Tukey's *post hoc* tests.
ANOVA, analysis of variance; DSS, dextran sodium sulfate; SCFA, short-chain fatty acid.
*** $P<.001$.

quence dataset has been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA707526.

2.6. Statistical analysis

Variables between groups were compared using the one-way analysis of variance with Tukey's *post hoc* tests. A two-tailed P value $<.05$ was considered statistically significant. Alpha diversity was measured using the Chao 1 richness index and the observed number of OTUs. Beta diversity was visualized through principal coordinate analysis to evaluate the dissimilarity between gut microbial communities. To explore the microbial strains associated with colon inflammation, Spearman's rank correlation coefficients were computed. All statistical procedures were performed using IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. DSS-induced colitis was not attenuated by oral administration of SCFAs

Figure 2 shows the impact of administration of SCFAs on DSS-induced colitis. The body weights of mice that received DSS were significantly decreased compared to the control mice. However, the body weights of the butyrate and SCFA mix groups were also reduced similarly to the DSS group (Fig. 2A).

Colon lengths were decreased in the DSS group compared to the controls (mean colon length: 5.55 cm *vs.* 8.17 cm, $P<.001$). However, there was no significant difference in colon length between the DSS, butyrate, and SCFA mix groups (mean colon length: DSS group, 5.55 cm; butyrate group, 5.50 cm; SCFA mix group, 5.15 cm) (Fig. 2B and C). Histological findings of the mouse colon tissue also revealed that colon inflammation was not significantly alleviated by SCFA supplementation. Histologic scores (range from 1 to 14, higher scores indicate more severe colonic inflammation) were not significantly different between the DSS, butyrate, and SCFA mix groups (mean histologic score: DSS group, 12.0; butyrate group, 10.83; SCFA mix group, 13.08) (Fig. 2D and E). Taken together, oral administration of butyrate or SCFA mixture did not significantly alleviate chemically induced murine colitis.

3.2. Administration of SCFAs regulated T cell differentiation

In FACS analysis, the expression of CD4+Foxp3+ regulatory T cells was numerically increased in the butyrate and SCFA mix

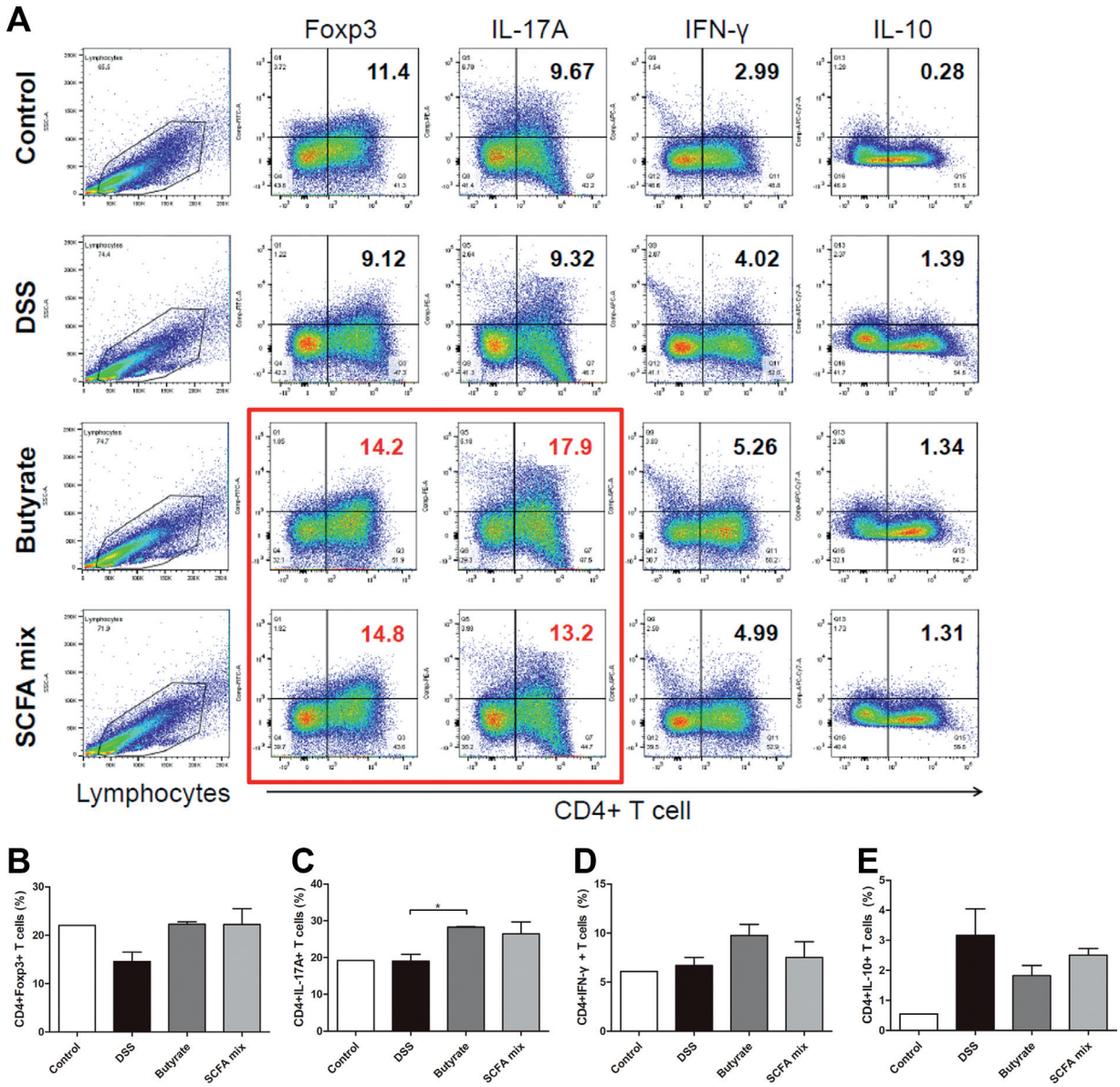

Fig. 3. Administration of SCFAs regulated T cell differentiation.
(A) Representative images of fluorescence-activated cell sorter analysis. Percentages of gated cells among total CD4+ T cells are presented within each box.
(B) Quantitative statistics demonstrate that the expression of CD4+Foxp3+ regulatory T cells were numerically increased in the butyrate and SCFA mix groups than in the DSS group, although this difference was not statistically significant (mean percentage of gated cells: DSS group, 14.55%; butyrate group, 22.25%; SCFA mix group, 22.20%).
(C) Compared to the DSS group, the expression of CD4+IL-17A-producing T cells was significantly increased in the butyrate group, but not significant in the SCFA mix group (mean percentage of gated cells: DSS group, 19.05%; butyrate group, 28.25%; SCFA mix group, 26.45% [P=.038 for DSS group *vs.* butyrate group]).
(D) The expression of CD4+IFN-γ-producing T cells was similar in all groups (mean percentage of gated cells: control, 6.10%; DSS group, 6.70%; butyrate group, 9.75%; SCFA mix group, 7.50%).
(E) The expression of CD4+IL-10-producing T cells was numerically higher in the DSS group than in the control group, but it was not statistically significant, and there were no significant differences in CD4+IL-10-producing T cell expressions between the DSS, butyrate, and SCFA mix groups (mean percentage of gated cells: control, 0.55%; DSS group, 3.17%; butyrate group, 1.83%; SCFA mix group, 2.51%).
Data were shown as mean with standard errors. Data for graphs were calculated using one-way ANOVA with Tukey's *post hoc* tests.
ANOVA, analysis of variance; DSS, dextran sodium sulfate; SCFA, short-chain fatty acid.
* P<.05.

groups than in the DSS group, although this difference was not statistically significant (mean percentage of gated cells: DSS group, 14.55%; butyrate group, 22.25%; SCFA mix group, 22.20%) (Fig. 3B). Compared to the DSS group, the expression of CD4+IL-17A-producing T cells was significantly increased in the butyrate group, but not significant in the SCFA mix group (mean percentage of gated cells: DSS group, 19.05%; butyrate group, 28.25%; SCFA mix group, 26.45% [P=.038 for DSS group *vs.* butyrate group]) (Fig. 3C).

The expression of IFN-γ-producing CD4+ T cells was similar in all groups (mean percentage of gated cells: control, 6.10%; DSS group, 6.70%; butyrate group, 9.75%; SCFA mix group, 7.50%) (Fig. 3D). The expression of IL-10-producing CD4+ T cells was numerically higher in the DSS group than in the control group, but it was not statistically significant, and there were no significant differences in CD4+IL-10+ T cell expressions between the DSS, butyrate, and SCFA mix groups (mean percentage of gated cells:

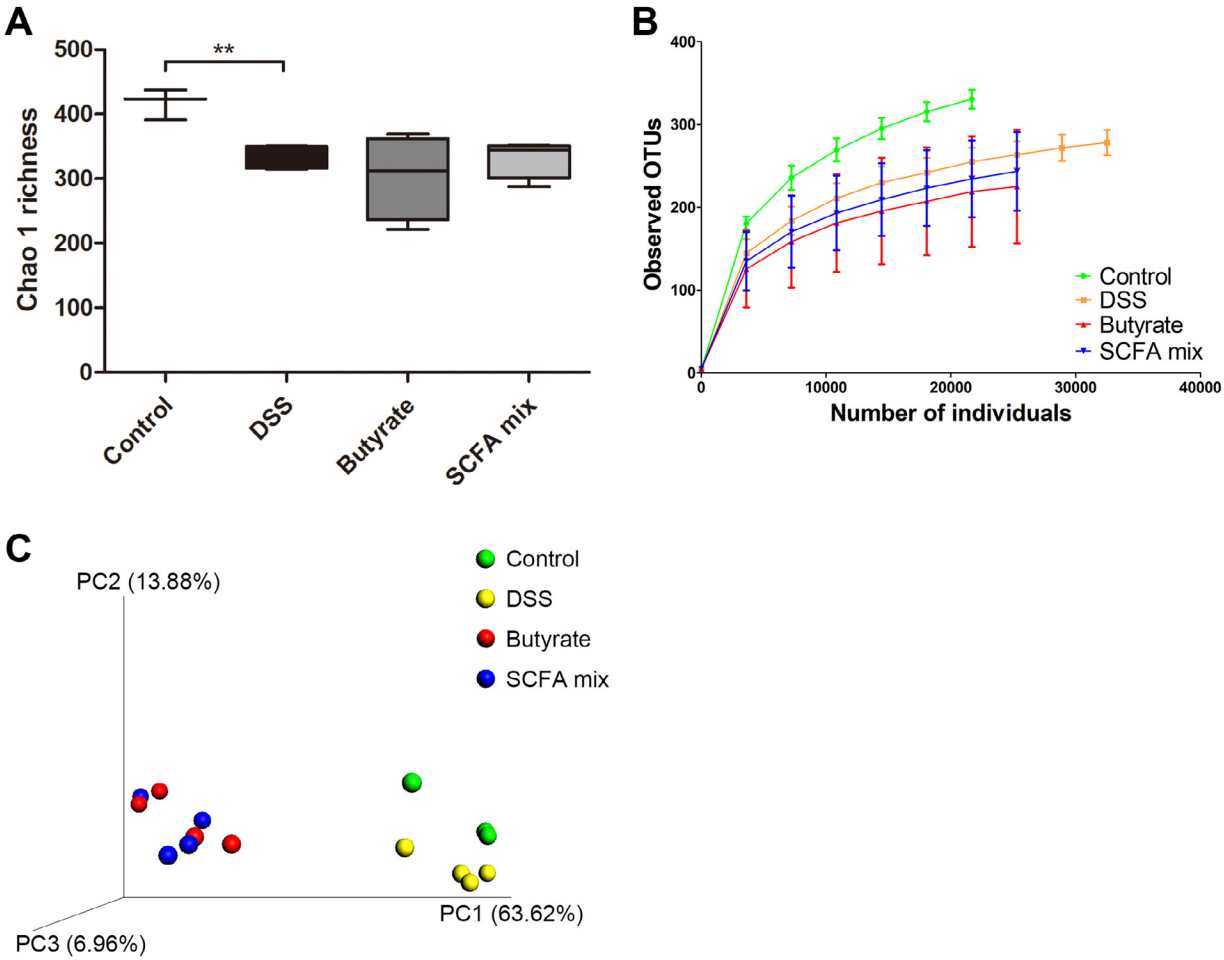

Fig. 4. SCFA supplementation led to distinct gut microbial community structures.
(A) Chao1 species richness indices were reduced in the DSS group than in the control (median 423.71 *vs.* 334.38, *P*=.003), but there were no significant differences between the DSS, butyrate, and SCFA mix groups (median 334.38 *vs.* 311.98 *vs.* 344.07, respectively). Data were presented as box and whisker plots.
(B) Rarefaction curves of observed OTUs demonstrate that microbial richness was decreased in the DSS group as compared with the control, and this decrease was similar among the DSS, butyrate, and SCFA mix groups. Error bars represent 95% confidence intervals.
(C) Principal coordinate analysis shows distinct microbial structure between the DSS group and SCFA groups.
Data for graphs were calculated using one-way ANOVA with Tukey's *post hoc* tests.
ANOVA, analysis of variance; DSS, dextran sodium sulfate; OTU, operational taxonomic unit; SCFA, short-chain fatty acid.
** $P<.01$.

control, 0.55%; DSS group, 3.17%; butyrate group, 1.83%; SCFA mix group, 2.51%) (Fig. 3E).

These results demonstrated that administration of SCFAs resulted in changes in T cell differentiation.

3.3. SCFA supplementation led to distinct gut microbial community structures

Figure 4 shows the changes in the diversity of gut microbiota associated with SCFA administration. Chao1 richness index was significantly lower in the DSS group than in the control (median 423.71 *vs.* 334.38, *P*=.003), but there were no significant differences between the DSS, butyrate, and SCFA mix groups (median 334.38 *vs.* 311.98 *vs.* 344.07, respectively) (Fig. 4A). Rarefaction curves of observed OTUs revealed reduced microbial richness in the DSS group as compared with the control, and this decrease was similar among the DSS, butyrate, and SCFA mix groups (Fig. 4B). However, principal coordinate analysis showed distinct gut microbial community structures between the DSS group and the butyrate and SCFA mix groups (Fig. 4C).

These results showed that SCFA supplementation did not restore microbial diversity within the group, but it altered the gut microbial community structure.

3.4. SCFA supplementation altered the gut microbiota composition

Figure 5 shows the relative abundance of gut microbiota in each group. At the phylum level, the butyrate and SCFA mix groups had higher relative abundances of Verrucomicrobia and Proteobacteria and lower relative abundances of Bacteroidetes and Firmicutes as compared with the control and DSS groups (Fig. 5A). The DSS group had a decreased Firmicutes/Bacteroidetes ratio as compared with the control (0.38 *vs.* 0.52), but the ratio was similar among the control, butyrate and SCFA mix groups (0.52 *vs.* 0.55 *vs.* 0.55, respectively) (Fig. 5B).

Comparison of the ten most abundant species revealed that the relative abundances of *Akkermansia muciniphila* and *Escherichia fergusonii* were increased in the butyrate and SCFA mix groups relative to the control and DSS groups. The relative abundance of *Bacteroides vulgatus* was decreased in the butyrate and SCFA mix groups compared to the control and DSS groups (Fig. 5C).

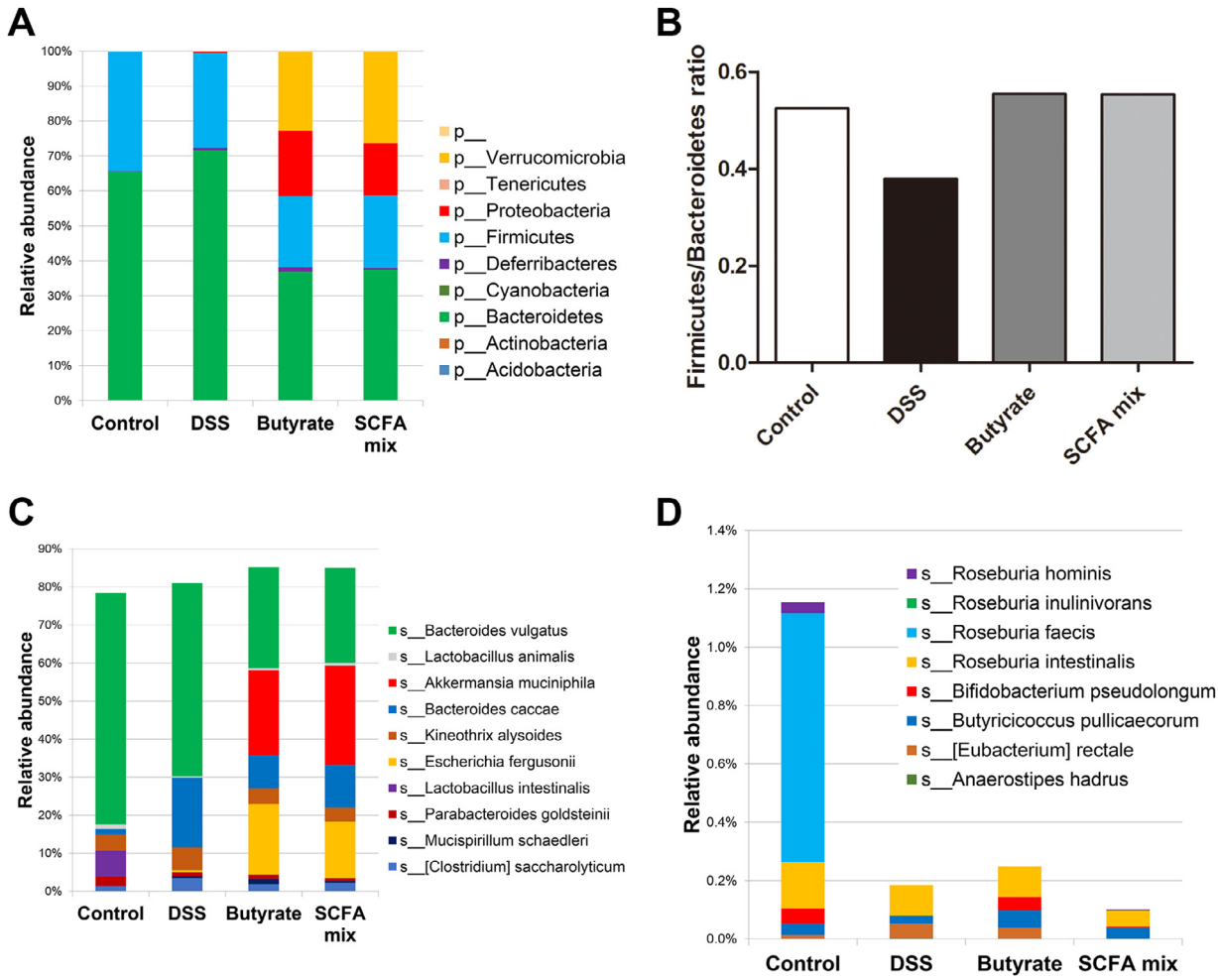

Fig. 5. SCFA supplementation altered the gut microbiota composition.
(A) At the phylum level, relative abundances of Verrucomicrobia and Proteobacteria were increased in the butyrate and SCFA mix groups. On the other hand, relative abundances of Bacteroidetes and Firmicutes were decreased in the butyrate and SCFA mix groups.
(B) The Firmicutes/Bacteroidetes ratio was decreased in the DSS group compared to the control group (0.38 *vs.* 0.52), but the ratio was similar among the control, butyrate and SCFA mix groups (0.52 *vs.* 0.55 *vs.* 0.55, respectively).
(C) At the species level, the relative abundances of *Akkermansia muciniphila* and *Escherichia fergusonii* were increased in the butyrate and SCFA mix groups relative to the control and DSS groups. The relative abundance of *Bacteroides vulgatus* was decreased in the butyrate and SCFA mix groups compared to the control and DSS groups.
(D) Butyrate-producing bacteria were significantly reduced in the DSS group compared to the controls. The butyrate and SCFA mix groups also showed decreased abundances of butyrate-producing bacteria, as in the DSS group. In particular, relative abundances of *Roseburia faecis* and *R. hominis* were significantly reduced.

Figure 5D shows the relative abundances of eight known butyrate-producing bacteria (*Roseburia hominis, R. inulinivorans, R. faecis, R. intestinalis, Bifidobacterium pseudolongum, Butyricicoccus pullicaecorum, Eubacterium rectale, Anaerostipes hadrus*). The relative abundance of butyrate-producing bacteria was significantly reduced in the DSS group as compared with the control. The butyrate and SCFA mix groups also showed a decreased abundance of butyrate-producing bacteria, as seen in the DSS group. In particular, the amounts of *R. faecis* and *R. hominis* were significantly reduced.

Taken together, these results showed that administration of SCFAs altered the gut microbial community, increased both protective and aggressive microbes, and did not affect the abundance of butyrate-producing bacteria.

3.5. Gut microbiota associated with colon inflammation

Table 1 shows the results of the Spearman correlation analyses for gut microbiota associated with colon inflammation. Correlation coefficients between the relative abundance of each microbiota and histologic inflammation score were analyzed. The relative abundance of genus *Roseburia*, a known butyrate producer, showed a negative correlation with histologic score (correlation coefficients [rho], -0.549; *P*=.034). Genus *Lactobacillus* also had a negative correlation with the degree of colitis (rho, -0.639; *P*=.010).

Phylum Proteobacteria was found to have a positive correlation with colon inflammation (rho, 0.534; *P*=.040). Genus *Escherichia* (rho, 0.532; *P*=.041) and genus *Mucispirillum* (rho, 0.556; *P*=.031) also showed positive correlation with colon inflammation.

4. Discussion

Oral administration of butyrate or a mixture of SCFAs did not alleviate DSS-induced colitis, but altered T cell differentiation and gut microbial profiles. SCFA supplementation induced expression of Foxp3+ regulatory T cells and IL-17-producing T cells, and increased the abundance of both protective and aggressive gut microbes.

Table 1
Gut microbiota associated with colon inflammation

	Correlation coefficients (rho)	*P* value
Negative correlation		
Roseburia genus	-0.549	.034
Roseburia hominis	-0.606	.017
Roseburia faecis	-0.697	.004
Lactobacillales order	-0.639	.010
Lactobacillus genus	-0.639	.010
Lactobacillus reuteri	-0.587	.021
Lactobacillus johnsonii	-0.697	.004
Positive correlation		
Proteobacteria phylum	0.534	.040
Escherichia genus	0.532	.041
Escherichia fergusonii	0.532	.041
Deferribacteres phylum	0.556	.031
Mucispirillum genus	0.556	.031
Mucispirillum schaedleri	0.556	.031

Correlation coefficients between the relative abundance of each microbiota and histologic inflammation score were analyzed using Spearman correlation analysis.

Unlike previous studies on SCFAs using experimental colitis models [19,20], our results did not show a significant alleviation of colon inflammation by oral administration of butyrate or a mixture of SCFAs. These conflicting results may have resulted from different study designs, but other causes need to be discussed.

First, SCFA supplementation may not be effective due to the impaired butyrate metabolism in inflamed intestinal mucosa. Patients with ulcerative colitis have impaired butyrate uptake and utilization, especially in the inflamed mucosa, and thereby the intracellular availability of butyrate in colonocytes is reduced [21]. Other studies have reported that the expression of genes involved in the butyrate oxidation pathway and butyrate synthesis by gut microbes is impaired in patients with IBD, and reduced butyrate synthesis gene expression was associated with more severe disease [22,23]. In addition, it has been reported that the anti-inflammatory response by butyrate is impaired in patients with active ulcerative colitis in part because the expression of genes involved in the inflammatory pathway is not strongly downregulated by butyrate in inflamed intestinal mucosa [24].

Another possible explanation is that butyrate may adversely affect wound healing in the intestinal mucosa and have an ambivalent effect on colon inflammation. Butyrate can suppress intestinal stem cell proliferation in the crypt base when intestinal mucosa is injured and the overlying colonocyte is damaged, resulting in a negative effect on wound healing in the short term [25]. In addition, a previous study reported that increased levels of butyrate with a fiber-rich diet paradoxically enhanced the cytotoxic ability of Shiga toxin and increased susceptibility to Shiga toxin-producing *E. coli* infections in the mouse model [26]. Furthermore, increased levels of butyrate with a fiber-rich diet have been reported to aggravate colon inflammation dependent on NOD-like receptor protein 3, suggesting that butyrate may serve as a fuel for colon inflammation [27]. These results suggest that SCFAs not only have the potential to alleviate colon inflammation but also exacerbate colitis. Fermentable fibers, gut microbiota, and their products (SCFAs) may be complexly implicated in gut inflammation, and appear to have both negative and positive impacts on gut inflammation depending on the specific conditions of the intestinal environment.

Changes in the expression of CD4+ T cells may also have contributed to the results that failed to alleviate colon inflammation. CD4+ T cells are divided into subsets, including Th1, Th2, Th17, and regulatory T cells, in the adaptive immune system, and naturally occurring CD4+CD25+ regulatory T cells play an important role in maintaining intestinal homeostasis [28,29]. In addition, the balance between Th17 and regulatory T cells is critical for regulation of the immune response [30]. Foxp3 plays a crucial role in regulating CD4+CD25+ regulatory T cell development and function [31]. CD4+CD25+Foxp3+ regulatory T cells are a component of the immune system designed to prevent excessive immune responses, and Foxp3 gene mutations have been associated with several autoimmune diseases [32,33]. Previous studies have shown that butyrate enhances the expression of Th1 cells and regulatory T cells and inhibits the expression of Th17 cells and IL-17, thereby alleviating colitis [34,35]. Butyrate-producing bacteria, including *Faecalibacterium prausnitzii* and *R. intestinalis*, have been associated with increased expression of the Foxp3 gene, inhibition of the Th17 pathway, and attenuation of colon inflammation [36,37]. The mechanism behind the increased expression of IL-17-producing T cells in our results, contrary to the results of previous studies, is unclear, but altered gut microbiota may be involved. The relative abundance of *E. fergusonii*, which shares the virulence factor of *E. coli*, was increased in the mice that received SCFA supplementation in our results [38]. A previous study on the virulence of *E. coli* have demonstrated that *E. coli* enhances Th1 and Th17 immune responses [39]. Furthermore, adherent-invasive *E. coli*, which can adhere to and invade intestinal epithelial cells, has been associated with increased expression of IL-17 and exacerbation of colon inflammation [40]. Therefore, a plausible explanation may be that increased *E. fergusonii* by administration of SCFAs activates the Th17 pathway and hinders alleviation of colon inflammation.

Another possible reason for why a significant alleviation of colitis did not occur in our study may be the unchanged IL-10-producing T cell expression by SCFA administration. A previous study has reported that the expression of Foxp3+ regulatory T cells is increased in patients with IBD, but the healing process of colitis is dependent on IL-10-producing CD4+ T cells [17]. In our results, SCFA supplementation did not lead to the increased expression of IL-10-producing T cells, which may contribute to the failure to attenuate colon inflammation significantly.

The administration of SCFAs was associated with a significant change in gut microbiota in our results. First, the relative abundance of the phylum Verrucomicrobia was increased in the SCFA mix and butyrate groups. A high abundance of phylum Verrucomicrobia has been observed in the healthy gut [41]. *A. muciniphila*, which belongs to phylum Verrucomicrobia, is an anaerobic, Gram-negative, mucin-degrading bacteria. In humans, *A. muciniphila* is found in the most healthy intestines, where it represents 1–4% of the total gut microbiota [42]. In contrast, the abundance of *A. muciniphila* is decreased in patients with IBD [43]. Fiber-rich diets or butyrate supplementation have been associated with increased abundance of *A. muciniphila* in previous studies [44,45]. Our results also revealed that the administration of SCFAs was associated with an increased abundance of protective bacteria, including the phylum Verrucomicrobia and the species *A. muciniphila*, supporting the results of previous literature.

On the other hand, the increased abundance of the phylum Proteobacteria in mice that received SCFAs in our results requires another explanation. An excessive butyrate concentration in the gut and increased abundance of the phylum Proteobacteria has been associated with exacerbation of colitis [27]. In addition, previous studies have shown that an increased abundance of the phylum Proteobacteria was associated with some inflammatory diseases, colorectal cancer, and relapse of colitis after fecal transplantation in patients with ulcerative colitis [46,47]. Moreover, the abundance of *E. fergusonii* was increased in SCFA-treated mice in our results. *E. fergusonii*, which belongs to the phylum Proteobacteria, is a human

and animal pathogen that has virulence factors identical to that of *E. coli* [38]. In a previous study, the growth of *E. coli* in the ileum was promoted by the administration of SCFAs [48]. These results suggest that the administration of SCFAs may be involved in the increased abundance of aggressive gut microbiota, including phylum Proteobacteria and *E. fergusonii*, and thereby having a negative effect on the alleviation of colon inflammation.

However, it cannot be concluded that SCFA supplementation has no protective effects on colon inflammation based on the results of this study alone. A recent study demonstrated that gut microbiota-derived SCFAs promote production of IL-22, which is crucial for intestinal homeostasis, *in vitro* and in humans. SCFA supplementation also protected the mouse intestine from *Citrobacter rodentium* infection and inflammatory insult [49]. More researches are needed to clarify the effect of SCFAs on intestinal health considering the adequate dose, route, and timing of SCFA administration.

In conclusion, oral administration of SCFAs did not result in a significant reduction in colon inflammation, but it did affect immune cell differentiation and the composition of gut microbiota. SCFA supplementation promoted both regulatory T cell and IL-17-producing T cell expression, and increased the abundance of both protective and aggressive gut microbiota, resulting in a neutral effect on colon inflammation in the DSS-induced murine colitis model.

Author Contributions

Conceptualization, Dong Soo Han and Chang Soo Eun; Data curation, A-reum Lee and Su Vin Jo; Formal analysis, Jae Gon Lee and Jiyoung Lee; Methodology, A-reum Lee, Su Vin Jo, Chan Hyuk Park and Chang Soo Eun; Writing – original draft, Jae Gon Lee and Jiyoung Lee; Writing – review & editing, Jae Gon Lee, Jiyoung Lee, A-reum Lee, Su Vin Jo, Chan Hyuk Park, Dong Soo Han and Chang Soo Eun. All authors have approved the final draft of the manuscript.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2021.108926.

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