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ORIGINAL ARTICLE

Effect of *Clostridium butyricum* on High-Fat Diet-Induced Intestinal Inflammation and Production of Short-Chain Fatty Acids

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Abstract

Background/Aims A high-fat diet (HFD) can cause intestinal inflammation and alter the gut microbiota; probiotics, however, are known to have anti-inflammatory effects. This study aimed to investigate the response of rat colon to HFD and the effect of *Clostridium butyricum* on HFD-induced intestinal inflammation and production of short-chain fatty acids (SCFAs) according to sex.

Methods Male and female 6-week-old Fischer-344 rats were fed a chow diet or HFD for 8 weeks, and Biovita or three different concentrations of *C. butyricum* were orally gavaged. The levels of tight junction proteins (TJPs), inflammatory markers in the ascending colonic mucosa, and bile acids (BAs) and SCFAs in stool were measured.

Results HFD significantly increased the histological inflammation scores and fat proportions. Fecal BA levels were higher in the HFD group than in the control group, with a more prominent increase in deoxycholic acid/cholic acid after probiotics administration in females; however, no statistically significant differences were observed. TJPs showed an opposite response to HFD depending on sex, and tended to increase and decrease after HFD in males and females, respectively. The HFD-reduced TJPs were recovered by probiotics, with some statistical significance in females. HFD-decreased butyric acid in stools appeared to be recovered by probiotics in males, but not in females. The expression of inflammatory markers (TNF-α) was increased by HFD in males and decreased with medium-concentration probiotic supplementation. The opposite was observed in females. MPO was increased by HFD in both sexes and decreased by probiotic supplementation.

Conclusions The probiotic *C. butyricum* improved indicators of HFD-induced colonic inflammation such as levels of inflammatory markers and increased the production of SCFAs and the expression of TJPs. These effects tended to be more pronounced in male rats, showing sex difference.

Keywords *Clostridium butyricum* · Short-chain fatty acids · High-fat diet · Sex difference

Abbreviations

HFD	High-fat diet
C. butyricum	*Clostridium butyricum*
BA	Bile acid
TJP	Tight function protein
MPO	Myeloperoxidase
IL	Interleukin
TNF	Tumor necrosis factor
SCFA	Short-chain fatty acid
SPF	Specific-pathogen-free
PBS	Phosphate-buffered saline
CFU	Colony-forming unit

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ELISA	Enzyme-linked immunosorbent assay
RT-qPCR	Real-time quantitative polymerase chain reaction
H&E	Hematoxylin and eosin
CA	Cholic acid
CDCA	Chenodeoxycholic acid
DCA	Deoxycholic acid
IBS	Irritable bowel syndrome

Introduction

The gut microbiota affects host health and the immune system, and dysbiosis plays a role in the development of various diseases, including irritable bowel syndrome (IBS) and colorectal cancer [1, 2]. Microbiota composition can change with age and sex and also rapidly and directly in response to dietary changes [3–5]. Recent studies have reported the effect of a high-fat diet (HFD) on the composition of gut microbiota; HFD promoted inflammatory reactions in the colon and change the gut microbial composition [6–8]. Previously, our team observed that these changes were different according to age and sex, that is, exposure to HFD for 8 weeks decreased the species richness of microbiota (Chao1) and increased Firmicutes/Bacteroidetes ratio only in the aged rats, while the concentration of myeloperoxidase (MPO) in colon mucosa was increased by HFD only in old males, but not in females [9]. These results suggest that the gut microbiota are affected by various factors, such as diet, age, and sex.

Probiotics can change the composition and balance of the gut microbiota and inhibit micro-inflammation of the intestine through anti-inflammatory actions [10], probably through the production of short-chain fatty acids (SCFAs) such as butyric acid. In a recent study, supplementation with the probiotic *Clostridium butyricum* reduced lipid accumulation in the liver and serum, improved glucose tolerance and insulin sensitivity, and reversed HFD-induced colitis in HFD-induced obesity mice model [11]. Biovita 3 bacterial species complex (Ildong Pharmaceutical, Co., Ltd.) is a formal preparation consisting of three probiotic bacterial strains, *C. butyricum*, *Lactobacillus sporogenes*, and *Bacillus subtilis*, and six vitamins and minerals, but the main ingredient is thought to be *C. butyricum* [12].

We hypothesized that the probiotic *C. butyricum* can improve the colon microenvironment by increasing butyric acid levels, and such effects would vary depending on sex. Thus, this study aimed to evaluate the effect of Biovita and probiotic *C. butyricum* on HFD-induced intestinal inflammation and production of SCFAs in both sexes by measuring the levels of inflammatory markers, histological inflammation, levels of SCFAs, and bile acids (BAs), which are known to regulate mucosal homeostasis and inflammation [13] via interactions with cellular receptors and luminal bacteria [14]. In addition, changes in tight junction proteins (TJPs) according to HFD and probiotics with sex differences were determined, as the downregulation of TJPs during intestinal inflammation is known to cause gut barrier dysfunction [15].

Materials and Methods

Study Design

Male and female specific-pathogen-free (SPF) Fischer-344/NSIc rats (6-week-old) were used (Orient, Seoul, Korea) [9, 16]. The rats were bred under SPF conditions at 23 °C under a 12:12-h light–dark cycle. Rats were divided into groups and were fed ad libitum with two different commercial diets: chow diet and HFD (chow: 3.85 kcal/g; HFD: 5.24 kcal/g, Research Diets, Inc., New Brunswick, NJ, United States). The HFD group received more than 60% of calories through fat, and the detailed composition of each formula is shown in Table 1. Probiotics were administered orally gavage using zonde: control (phosphate-buffered saline; PBS), Biovita (PBS dissolved mixture of *L. sporogenes* IDCC 1201, *C. butyricum* IDCC 1301, and *B. subtilis* IDCC 1101, Ildong Pharmaceutical, Co., Ltd., Seoul, South Korea), or low, medium, and high-concentration of *C. butyricum* (freeze-dried bacteria *C. butyricum* IDCC 1301 dissolved in PBS at a concentration of 0.1 g/ml as a high-concentration and 10- and 100-fold dilutions as medium and low-concentrations, respectively). Probiotics were gavaged everyday including weekends and holidays, at 10 to 11 a.m., a standardized administration hour. The concentrations used were decided by referring to prior experiments [17, 18] conducted to determine dose dependency of these concentrations; the final concentrations of *C. butyricum* were 1.23×10^9 colony-forming units (CFU)/ml, 1×10^7 CFU/ml, 1×10^8 CFU/ml, and

Table 1 Composition of chow and high-fat diet

	Protein	Carbohydrate	Fiber	Fat	Mineral/vitamin	Groups fed
Chow diet	17.0	48.0	18.0	3.0	14.0	1, 2
High-fat diet	26.2	25.6	6.5	34.9	6.8	3–12

Represented by weight (%)

1×10^9 CFU/ml in Biovita, low-concentration *C. butyricum*, medium-concentration *C. butyricum*, and high-concentration *C. butyricum* groups, respectively.

The diet and administration of probiotics in each group are shown in Fig. 1. A total of 96 rats were used, and the number of rats in each group was as follows: male with chow diet (M. C, $n = 8$), female with chow diet (F. C, $n = 8$), male with HFD (M. HF, $n = 8$), female with HFD (F. HF, $n = 8$), male with HFD and Biovita (M. Bio, $n = 8$), female with HFD and Biovita (F. Bio, $n = 8$), male with HFD and low-concentration *C. butyricum* (M. LCB, $n = 8$), female with HFD and low-concentration *C. butyricum* (F. LCB, $n = 8$), male with HFD and medium-concentration of *C. butyricum* (M. MCB, $n = 8$), female with HFD and low-concentration of *C. butyricum* (F. MCB, $n = 8$), male with HFD and high-concentration *C. butyricum* (M. HCB, $n = 8$), and female with HFD and high-concentration *C. butyricum* (F. HCB, $n = 8$).

During the 8 weeks of feeding chow or HFD, the food intake and body weight of each rat were measured weekly. After the feeding period, terminal anesthesia was induced via inhalation of carbon dioxide. Feces, blood, and colon tissues were obtained and stored at − 80℃ immediately for further analysis. Tissue samples were fixed in 10% buffered formalin immediately, and after that, treated at room temperature and entrusted to a specialized company. This study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of South Korea. The protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital (Permission No. BA1506-178/027-01).

Histological Examinations Using Hematoxylin–Eosin Staining

To evaluate the levels of fat accumulation and inflammation in the colonic mucosa, histological analysis of the ascending colon was performed. For tissue preparation, 1 cm was removed from the cecum and anus, and 1 cm of the proximal part of the colon was collected [9]. Samples were fixed in 10% buffered formalin. Tissue specimens embedded in paraffin blocks were cut perpendicular to the lumen into 4-mm-thick sections and stained with hematoxylin and eosin (H&E). Three H&E-stained slides per rat and four fields per slide were randomly selected. The fat tissue and total smooth muscle areas were quantified using the ImagePro Plus analysis system (Media Cybernetics, Inc., San Diego, CA, United States). The fat proportion is described as the percentage area of fat to that of total smooth muscle [9, 16]. Histological scoring was performed by an experimenter who was blinded to the identities of the samples. Colonic infiltration by inflammatory cells was scored as previously described [19]. Briefly, colonic epithelial damage was scored as 0 for normal; 1 for hyperproliferation, irregular crypts, and goblet cell loss; 2 for mild-to-moderate crypt loss (10–50%); 3 for severe crypt loss (50–90%); 4 for complete crypt loss and intact surface epithelium; 5 for small- to medium-sized

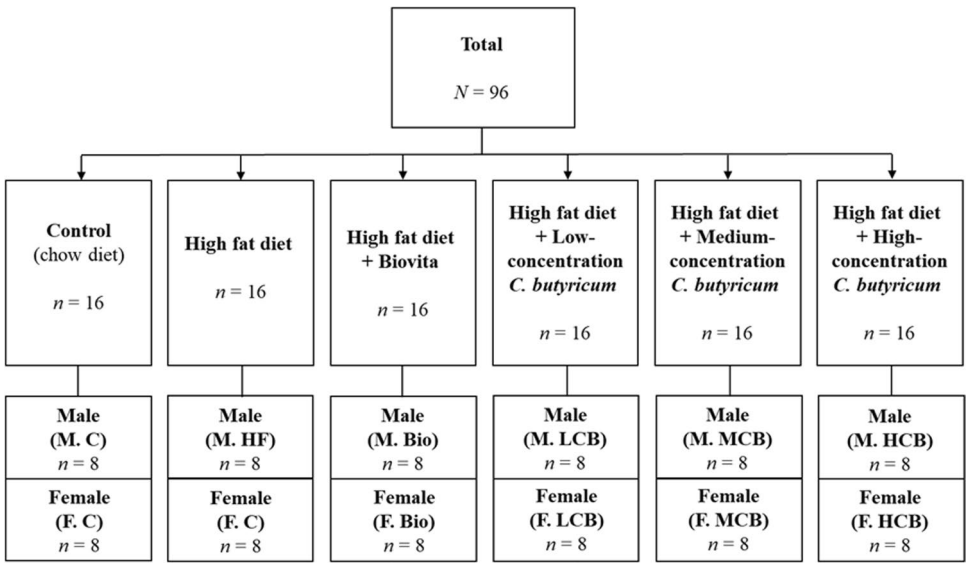

Fig. 1 Study flow chart demonstrating each study group. A total of 12 groups were formed and classified by sex, diet, and probiotics gavaged. *M. C* male control group, *M. HF* male high-fat diet group, *M. Bio* male Biovita group, *M. LCB* male low-concentration *C. butyricum* group, *M. MCB* male medium-concentration *C. butyricum* group, *M. HCB* male high-concentration *C. butyricum* group, *F. C* female control group, *F. HF* female high-fat diet group, *F. Bio* female Biovita group, *F. LCB* female low-concentration *C. butyricum* group, *F. MCB* female medium-concentration *C. butyricum* group, *F. HCB* female high-concentration *C. butyricum* group

ulcers (< 10 crypt widths); and 6 for large ulcers (≥ 10 crypt widths). Inflammatory cell infiltration was also scored for the mucosa (0 = normal, 1 = mild, 2 = moderate, 3 = severe), submucosa (0 = normal, 1 = mild to modest, 2 = severe), and muscle/serosa (0 = normal, 1 = moderate to severe). The sum of the scores for colonic epithelial damage and inflammatory cell infiltration was calculated for each slide (three slides per rat) to generate a score of 0–12. The average score for the three slides was used as the score for each rat.

Measurements of the Concentration of BAs and SCFAs

To measure the concentrations of SCFAs and BAs, feces were collected immediately after defecation, after rats were fed a control diet or a HFD with probiotic administration for 8 weeks. All fecal samples were immediately frozen in liquid nitrogen and stored at − 80 °C. Frozen feces were homogenized in 2 ml of ice-cold PBS using a vortex and incubated at 4 °C for 20 min. After incubation, the homogenates were centrifuged at 12,000 × *g* for 20 min and the supernatants were transferred to a fresh tube. The BA in feces was measured from the supernatant using enzyme-linked immunosorbent assay (ELISA) kits (Chenodeoxycholic acid ELISA kit, Cholic acid ELISA kit, Cell Biolabs, Inc., San Diego, CA, USA; Deoxycholic acid ELISA kit, Bluegene, Shanghai, China), following the manufacturer's recommendations. Acetic acid and butyric acid in each sample were separated and measured using an Agilent 1100 series instrument (Agilent, CA, USA) equipped with a C_{18} column (ZORBAX Eclipse XDB-C_{18}, analytical 4.6 * 150 mm, 5-Micron, Agilent, CA, USA) and a UV detector (210 nm). The mobile phase consisted of 90% 10 mM KH_2PO_4 and 10% acetonitrile, in the same manner as in previous studies by our team [20].

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) and Enzyme-Linked Immunosorbent Assay (ELISA)

Scraped ascending colonic mucosa samples were homogenized in lysis buffer and centrifuged. The lysis buffer was composed of radioimmunoprecipitation assay buffer, proteinase inhibitors, and phosphatase inhibitors. The supernatant was used for the analysis.

Total RNAs was extracted from the ascending colonic mucosa using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), as recommended by the manufacturer, and the collected RNA was purified using RNeasy mini kits (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using 1 µg of total RNA with the High-Capacity cDNA kit (Applied Biosystems™, Waltham, Massachusetts, USA), according to the manufacturer's instructions. RT-qPCR was performed using a StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with Power SYBR™ Green PCR Master Mix (Applied Biosystems™, Waltham, Massachusetts, USA) according to the manufacturer's instructions and protocols. The mRNA expression of inflammatory markers interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, anti-inflammatory marker IL-10, and TJPs claudin-1 (CLDN1), CLDN2, CLDN4, occludin (OCLN), and zonula occludens 1 (ZO1) was measured by RT-qPCR. The primer sequences are presented in Table 2. The expression levels of mRNA from the target genes were compared with those of the endogenous control β-actin using the $2^{-\Delta\Delta C_t}$ method. MPO was measured using an ELISA kit (HyCult Biotech, Uden, Netherlands) according to the manufacturer's instructions.

Statistical Analysis

The body weights, histology data, and measured values of ELISA and RT-qPCR were compared using the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test with Holm–Bonferroni correction which are used for non-parametric test because the size of sample was less than 30 in each groups. These analyses were performed using SPSS version 18 (IBM Corp, Armonk, NY, United States), and statistical significance was set at $P < 0.05$.

Table 2 The sequences of primers used for the real-time PCR

Gene		Primer sequence
IL-1β	Forward	5′-GCA TCC AGC TTC AAA TCT CA-3′
	Reverse	5′-ATC ATC CCA CGA GTC ACA GA-3′
TNF-α	Forward	5′-GCC GAT TTG CCA TTT CAT AC-3′
	Reverse	5′-TGG AAG ACT CCT CCC AGG TA-3′
IL-6	Forward	5′-CCG GAG AGG AGA CTT CAC AG-3′
	Reverse	5′-CAG AAT TGC CAT TGC AAC AAC-3′
IL-10	Forward	5′-CCG GAG AGG AGA CTT CAC AG-3′
	Reverse	5′-CAG AAT TGC CAT TGC AAC AAC-3′
Claudin-1	Forward	5′-CAT GAA GTG CAT GAG ATA CT-3′
	Reverse	5′-ATA TTA TGC CCC CGA TGA CA-3′
Claudin-2	Forward	5′-GTG GCT GTA GTG GGT GGA GT-3′
	Reverse	5′-CCT GAG GTG AGC AGG AAA AG-3′
Claudin-4	Forward	5′-GAT GGT CAT CAG CAT CAT CG-3′
	Reverse	5′-GTC TCG TCC TCC ATG CAG TT-3′
Occludin	Forward	5′-TTT TGC TTC ATC GCT TCC TT-3′
	Reverse	5′-CAG GAT TGC GCT GAC TAT GC-3′
ZO-1	Forward	5′-GCT CAC CAG GGT CAA AAT GT-3′
	Reverse	5′-GGC TTA AAG CTG GCA GTG TC-3′

Results

Changes in Body Weight and Caloric Intake

Figure 2 shows the calorie intake (A, B) and the body weight changes (C, D) in rats during the feeding period. The average daily calorie intake in males was increased in HFD group, while M. HCB showed significantly lower calorie intake compared to M. HF ($P=0.043$), and F. LCB showed significantly lower calorie intake compared to F. HF ($P=0.002$). In spite of no difference in female calorie intake, both sexes gained weight steadily; however, the pattern was different. In males, the weight gain was in the order of M. HF, M. C, M. Bio, M. LCB, M. MCB, and M. HCB, whereas in females, the order was: F. HF, F. LCB, F. Bio, F. C, F. MCB, and F. HCB.

Intestinal Mucosal Inflammation and Fat Proportion by Histology

Differences in the inflammation score and fat distribution in the pathological findings of the colonic mucosa were analyzed (Fig. 3). Inflammation scores were significantly higher in the HFD group than in the control group in both sexes (male control vs. HFD $P<0.001$; female control vs. HFD $P<0.001$). In males, the scores tended to be lower in the Biovita and *C. butyricum* groups than in the HFD groups, but the difference was not statistically significant (Fig. 3A). In females, the scores tended to be lower in the low- and medium-concentration *C. butyricum* groups than in the HFD group, but no significant differences were observed (Fig. 3B).

Fat proportions were also significantly higher in the HFD group than in the control group for both sexes (male control vs. HFD, $P=0.021$; female control vs. HFD, $P=0.021$). Further, fat proportions were lower in the low- and medium-concentration *C. butyricum* groups than in the HFD groups in males (Fig. 3C), but lower only in the low-concentration *C. butyricum* group in females (Fig. 3D). However, significant differences were not observed.

Figure 3E and F show the graphical data of the histopathology. Significant increase of intestinal mucosal inflammation in aspect of loss of crypt (blue box), and immune cell infiltration (black circle) and submucosal fat deposition (red arrows) after HFD were observed in males (Fig. 3E) as well as females (Fig. 3F); although no clear difference was detected depending on the concentration of *C. butyricum* administered. The average scores in HFD groups were about 2, which represents mild-to-moderate crypt loss, and when each score parameters were depicted separately, the main epithelial damage was found to be the loss of crypt. However, statistical significances among groups were not observed. Also, in aspect of inflammatory cell infiltration, most of H&E slides showed inflammatory cells in mucosa.

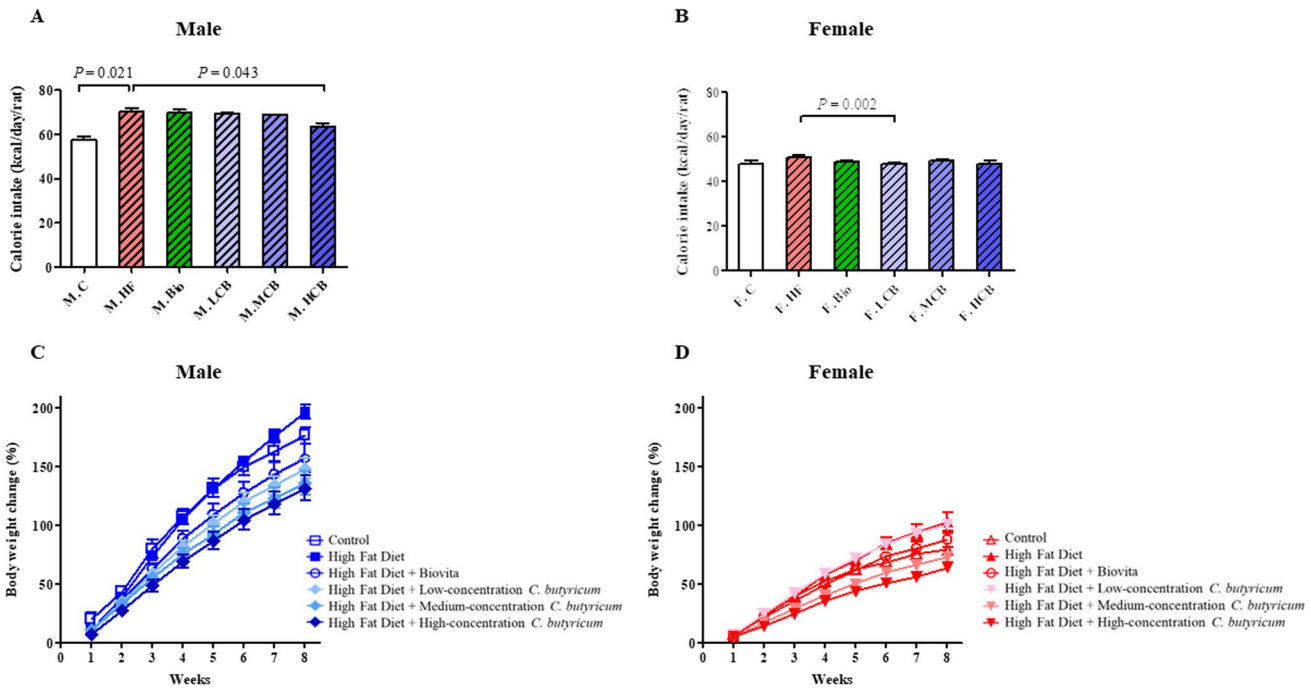

Fig. 2 Caloric intake and body weight change of male and female rats. Mean ± SEM, Mann–Whitney *U*-test. Calorie intake, M. C vs. M. HF $P=0.021$, M. HF vs. M. HCB $P=0.043$, F. HF vs. F. LCB $P=0.002$

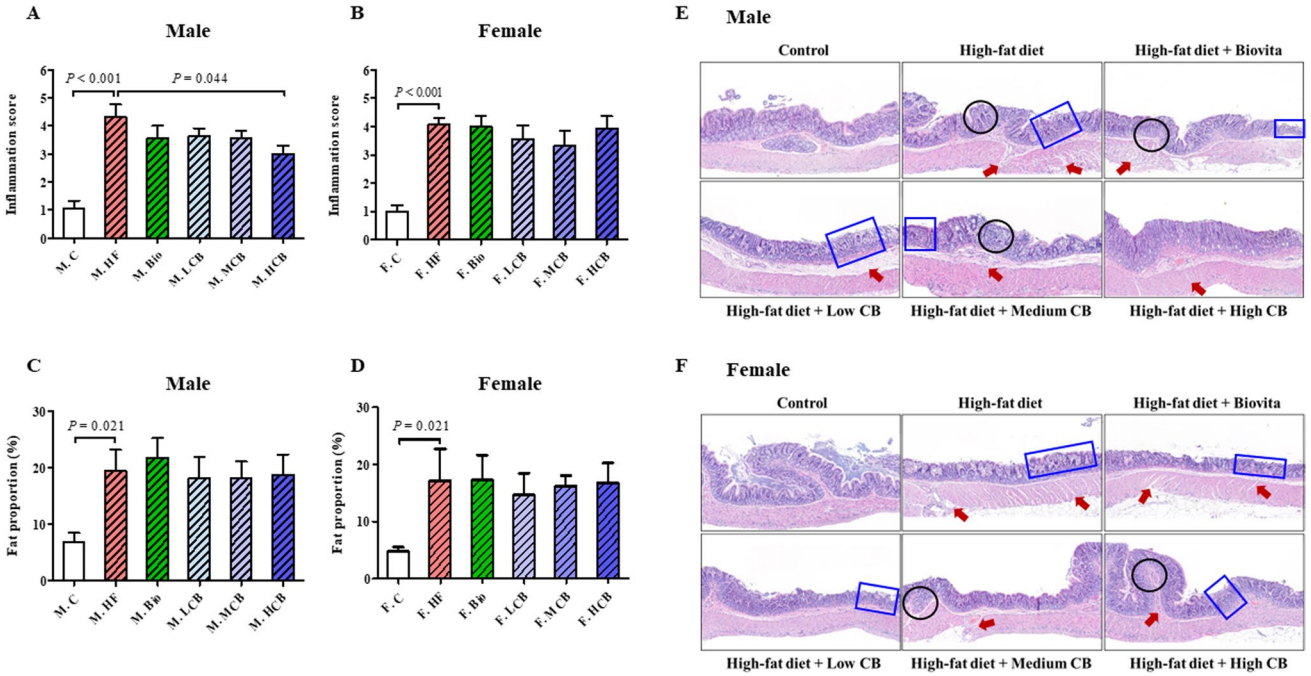

Fig. 3 Histological inflammation scores and fat proportions in male and female rats (**A**–**D**) exposed or not to HFD, Biovita, and *C. butyricum*, and sample of H&E-stained slides (**E**, **F**). Mean ± SEM, Mann–Whitney *U*-test. Inflammation score, M. C vs. M. HF $P < 0.001$, M. HF vs. M. HCB $P < 0.044$, F. C vs. F. HF $P < 0.001$; fat proportion, M. C vs. M. HF $P = 0.021$, F. C vs. F. HF $P < 0.021$. Increase of intestinal mucosal inflammation in aspect of loss of crypt (blue box), immune cell infiltration (black circle) and submucosal fat deposition (red arrows) after HFD were observed

Bile Acid Concentrations

Fecal levels of primary (cholic acid; CA, chenodeoxycholic acid; CDCA) and secondary (deoxycholic acid; DCA) BAs were measured to determine the change in BA composition according to the diet (Fig. 4). CDCA, CA, and DCA levels tended to be higher in the HFD group than in the control group in both males and females, but there were no statistical significances. The changes in CDCA, CA, and DCA levels by probiotics were not consistent and showed no clear trend.

The ratio of secondary to primary BA (DCA/CA) in each group was also calculated. DCA/CA tended to be lower in the HFD groups compared to the control groups in both males and females and the increase in DCA/CA by probiotics seemed to be more prominent in females, but no statistical significance was observed.

Levels of Intestinal TJPs

The mRNA expression of TJPs showed an opposite response to HFD in males and females, and the mRNA expression of all five TJPs increased after HFD in males but decreased after HFD in females (Fig. 5). The levels of CLDN1 were higher in the HFD group than in the control group ($P = 0.004$) and were higher in the Biovita, medium- and high-concentration *C. butyricum* groups than in the HFD group without statistical significance in males (Fig. 5A), while they were lower in the HFD group than in the control group ($P = 0.028$) and were higher in the Biovita, medium- and high-concentration *C. butyricum* groups than in the HFD group, with some statistical significance in females (HFD vs. high-concentration *C. butyricum* $P = 0.002$) (Fig. 5B). CLDN2 levels were higher in the HFD group than in the control group and were lower in the probiotic groups than in the HFD group (HFD vs. low-concentration *C. butyricum* $P = 0.011$) in males (Fig. 5C), whereas they were lower in the HFD group than in the control group ($P = 0.008$) and were higher in the probiotic groups than in the HFD group, with some statistical significance in females (HFD vs. high-concentration *C. butyricum* $P = 0.021$) (Fig. 5D). CLDN4 levels were higher in the HFD group than in the control group ($P = 0.004$) and were lower in the probiotic groups than in the HFD group (HFD vs. low-concentration *C. butyricum* $P = 0.011$) in males (Fig. 5E), whereas they were lower in the HFD group than in the control group ($P = 0.004$) and were higher in the probiotic groups than in the HFD group, with some statistical significance in females (HFD vs. Biovita $P = 0.046$, HFD vs. high-concentration *C. butyricum* $P = 0.046$) (Fig. 5F). The levels of OCLN were higher in the HFD group than in the control group and were lower in the probiotic groups than in the HFD group

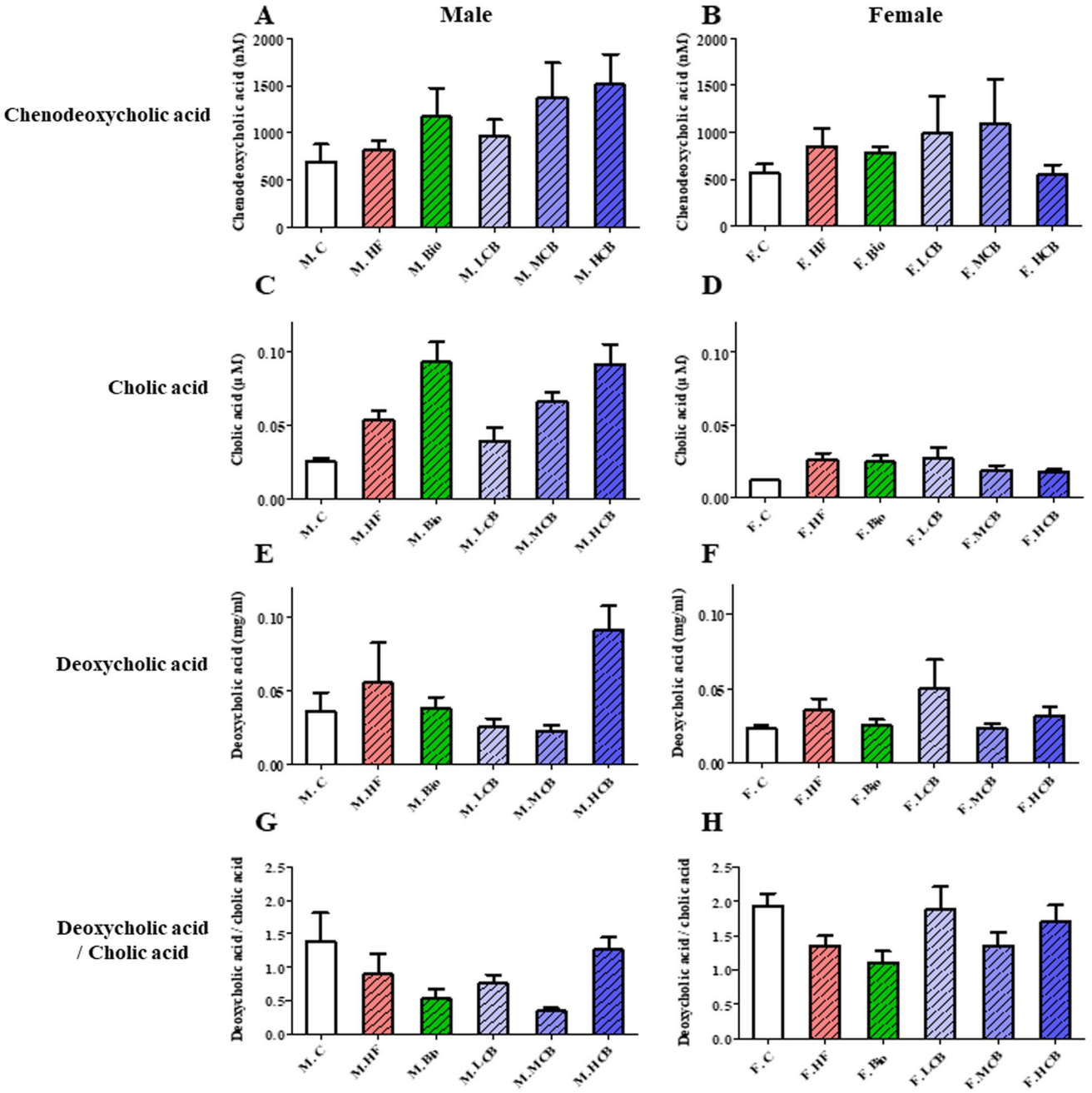

Fig. 4 Fecal bile acid concentration in male and female rats (**A**–**H**) exposed or not to HFD, Biovita, and *C. butyricum*. Mean ± SEM, Mann–Whitney *U*-test

(HFD vs. low-concentration *C. butyricum* $P = 0.010$) in males (Fig. 5G), while they were lower in the HFD group than in the control group ($P = 0.005$) and were higher in the Biovita, medium- and high-concentration *C. butyricum* groups than in the HFD group, with some statistical significance in females (HFD vs. high-concentration *C. butyricum* $P = 0.046$) (Fig. 5H). The levels of ZO1 were higher in the HFD group than in the control group ($P = 0.010$) and were lower in the probiotic groups than in the HFD group (HFD vs. low-concentration *C. butyricum* $P = 0.011$) in males (Fig. 5I), while they were lower in the HFD group than in the control group ($P = 0.008$) and were higher in the Biovita, medium- and high-concentration *C. butyricum* groups than in the HFD group, with some statistical significance in females (HFD vs. high-concentration *C. butyricum* $P = 0.036$) (Fig. 5J).

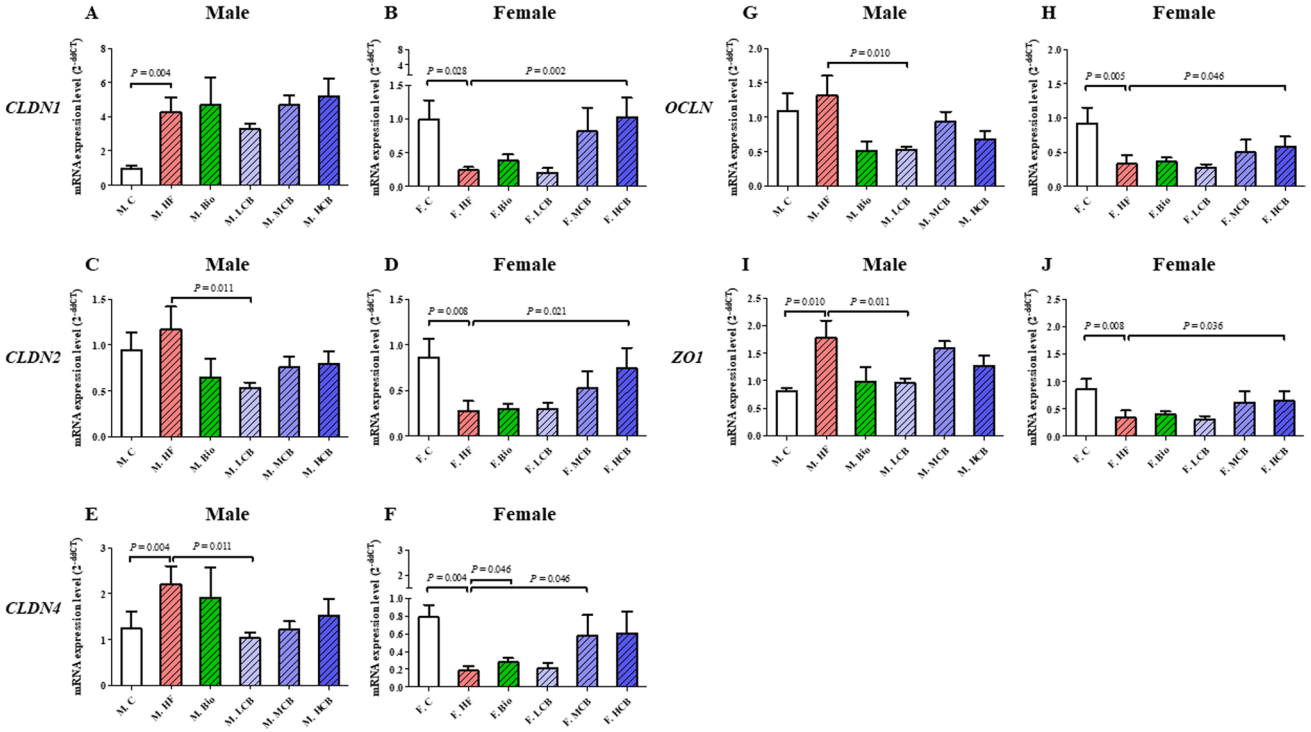

Fig. 5 Tight junction protein mRNA expression in male and female rats (**A**–**J**) exposed or not to HFD, Biovita, and *C. butyricum*. Mean ± SEM, Mann–Whitney *U*-test. CLDN1, M. C vs. M. HF $P=0.004$, F. C vs. F. HF $P=0.028$, F. HF vs. F. HCB $P=0.002$; CLDN2, M. HF vs. M. LCB $P=0.011$, F. C vs. F. HF $P=0.008$, F. HF vs. F. HCB $P=0.021$; CLDN4, M. C vs. M. HF $P=0.004$, M. HF vs. M. LCB $P=0.011$; OCLN, M. HF vs. M. LCB $P=0.010$, F. C vs. F. HF $P=0.005$, F. HF vs. F. HCB $P=0.046$; ZO1, M. C vs. M. HF $P=0.010$, M. HF vs. M. LCB $P=0.011$, F. C vs. F. HF $P=0.008$, F. HF vs. F. HCB $P=0.036$

Levels of Fecal SCFAs

In the fecal SCFA measurement, the levels of butyric acid and acetic acid in the HFD groups were lower than those in the control groups, with statistical significance in butyric acid levels (males and females, control vs. HFD, $P<0.001$). The levels of butyric acid were higher in the Biovita and *C. butyricum* groups than in the HFD group, with some statistical significance in males (HFD vs. low-concentration *C. butyricum* $P=0.012$; HFD vs. high-concentration *C. butyricum* $P=0.003$, Fig. 6A). A consistent pattern was not observed in females, and butyric acid levels were lower in the Biovita group and higher in the medium and high-concentration *C. butyricum* groups than in the HFD group, without statistical significance (Fig. 6B). Distinct differences in the levels of acetic acid among groups were not observed in males (Fig. 6C), and they tended to be lower in the Biovita and low-concentration *C. butyricum* groups than in the HFD group in females without statistical significance (Fig. 6D).

Levels of Inflammatory and Anti-inflammatory Markers by RT-qPCR and Protein Assay

The results of the inflammatory marker analysis are shown in Fig. 7. IL-1β expression was higher in the HFD group than in the control group, and was lower in the probiotic groups than in the HFD group without statistical significance in males (Fig. 7A), whereas it was lower in the HFD group than in the control group and was higher in the medium- and high-concentration *C. butyricum* groups than in the HFD group (Fig. 7B). IL-6 expression was higher in the HFD group than in the control group, and was higher in the medium- and high-concentration *C. butyricum* groups without statistical significance in males (Fig. 7C), whereas it was lower in the HFD group than in the control group ($P=0.021$) and was higher in the low-, medium-, and high-concentration *C. butyricum* groups than in the HFD group (Fig. 7D). TNF-α expression was higher in the HFD group than in the control group ($P=0.001$) and lower in the

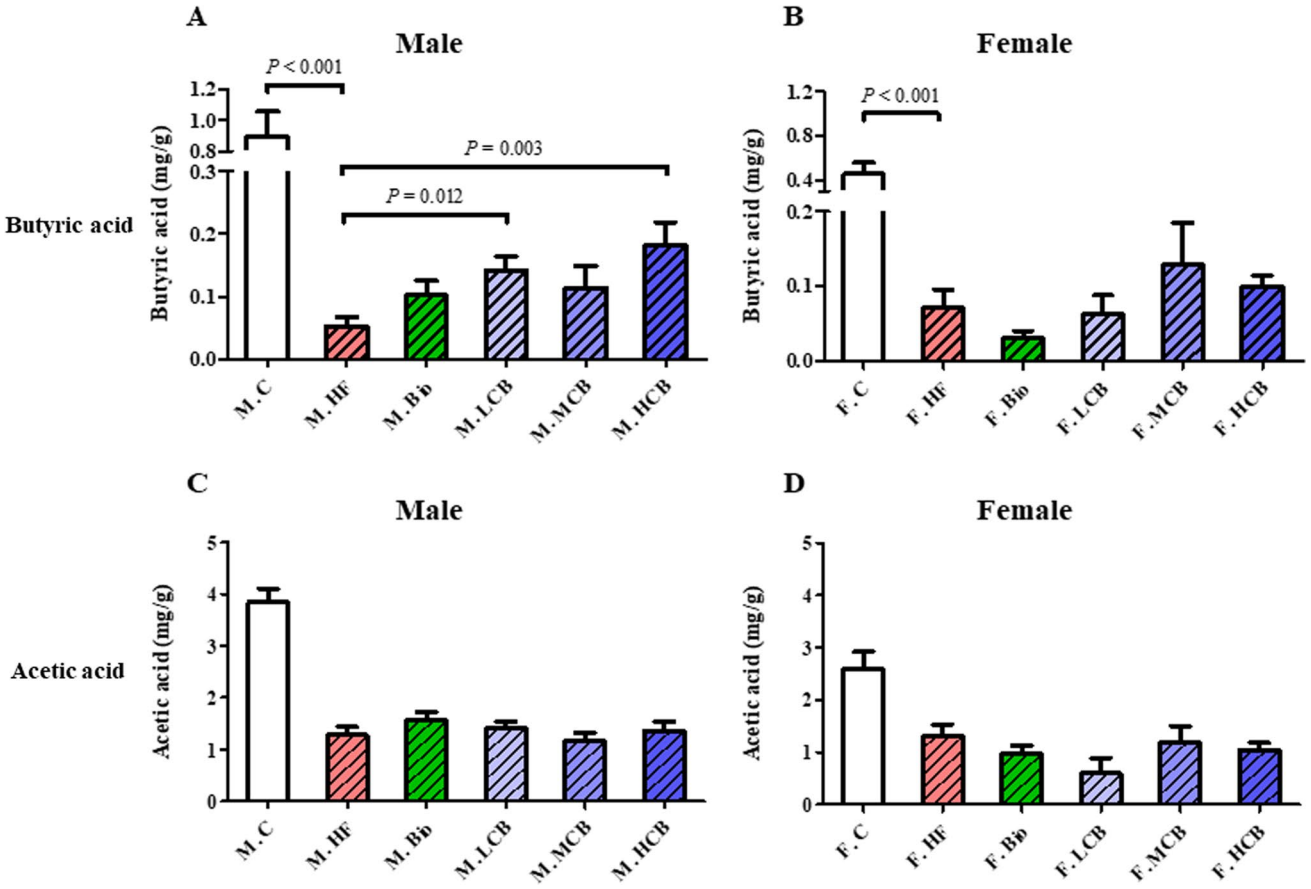

Fig. 6 Fecal short-chain fatty acids in male and female rats (**A**–**D**) exposed or not to HFD, Biovita, and *C. butyricum*. Mean ± SEM, Mann–Whitney *U*-test. Butyric acid, M. C vs. M. HF $P < 0.001$, M. HF vs. M. LCB $P = 0.012$, M. HF vs. M. HCB $P = 0.003$, F. C vs. F. HF $P < 0.001$

probiotics group than in the HFD group in males (Fig. 7E), whereas it was lower in the HFD group than in the HFD group ($P = 0.011$) and higher in the probiotic groups (HFD vs. high-concentration *C. butyricum* $P = 0.001$) (Fig. 7F). MPO levels were significantly higher in the HFD group than in the control group and lower in the probiotic groups than in the HFD group, with statistical significance in males (HFD vs. Biovita $P = 0.049$, HFD vs. high-concentration *C. butyricum* $P = 0.012$) (Fig. 7G), while were lower in the HFD group than in the control group and were lower in the low-concentration *C. butyricum* group ($P = 0.021$) but higher in high-concentration *C. butyricum* group than in the HFD group (Fig. 7H).

The expression of IL-10, an anti-inflammatory marker, was lower in the HFD group than in the control group and was higher in the Biovita and low- and medium-concentration *C. butyricum* groups and lower in high-concentration *C. butyricum* group, with some statistical significance in males (HFD vs. medium-concentration *C. butyricum* $P = 0.002$; medium-concentration *C. butyricum* vs. high-concentration *C. butyricum* $P = 0.006$; respectively) (Fig. 7I). It was lower in the HFD group than in the control group and was higher in the low- and medium-concentration *C. butyricum* groups than in the HFD group in females, but the difference was not statistically significant (Fig. 7J).

Discussion

Our study showed sex differences in the process of intestinal inflammation induced by HFD and in the response to probiotics. The increase in inflammatory marker levels was more pronounced in males, whereas the decrease in the expression of TJPs was more pronounced in females. Sex differences were also observed in the response to the probiotic *C. butyricum*. That is, the decrease in inflammatory marker levels, increase in anti-inflammatory marker levels, and increase in SCFA levels after administration of *C. butyricum* were more pronounced in males than in females. However, the increase in the expression of TJPs after the administration of *C. butyricum* was more pronounced in females than in males. Different weight gain patterns after HFD intake were also observed between males and females. For instance, the inhibition of weight gain by probiotics was more pronounced

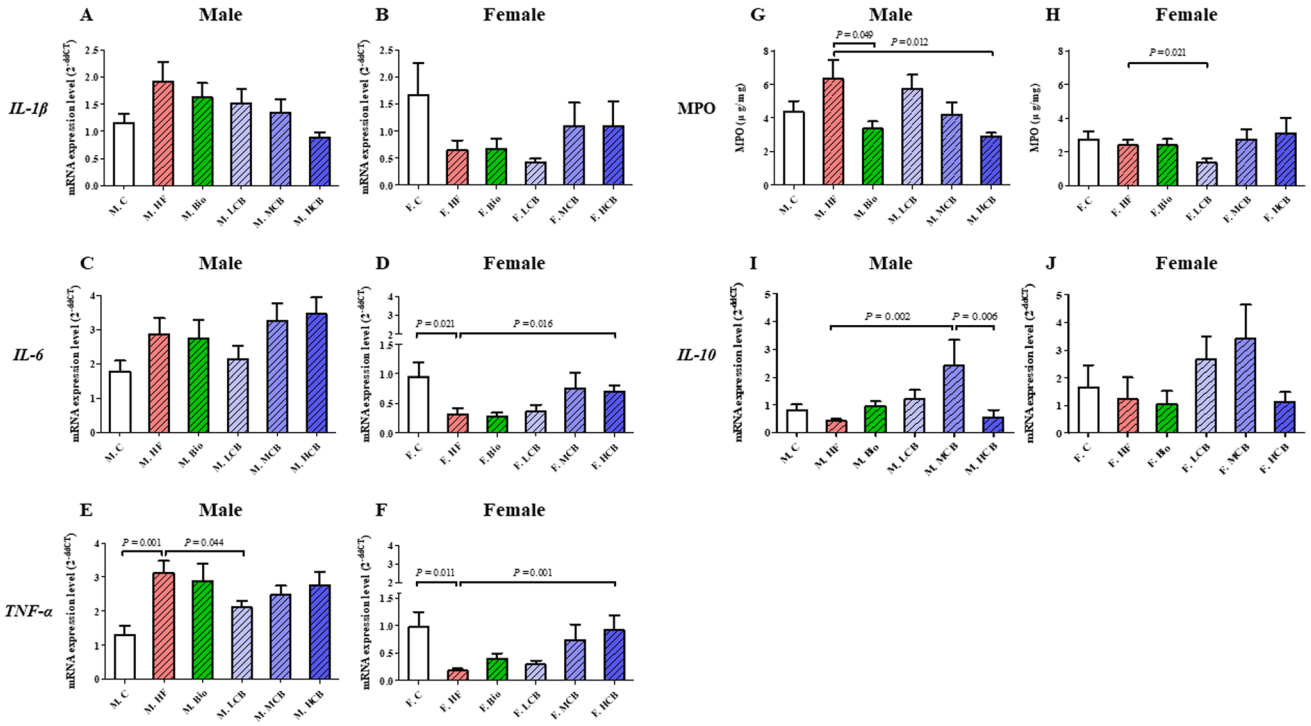

Fig. 7 Inflammatory marker protein level and mRNA expression in male and female rats (**A**–**J**) exposed or not to HFD, Biovita, and *C. butyricum*. Mean ± SEM, Mann–Whitney *U*-test. IL-6, F. C vs. F. HF $P = 0.021$, F. HF vs. F. HCB $P = 0.016$; TNF-α, M. C vs. M. HF $P = 0.001$, M. HF vs. M. LCB $P = 0.044$, F. C vs. F. HF $P = 0.011$, F. HF vs. F. HCB $P = 0.001$; MPO, M. HF vs. M. Bio $P = 0.049$, M. HF vs. M. HCB $P = 0.012$, F. HF vs. F. LCB $P = 0.021$; IL-10, M. HF vs. M. MCB $P = 0.002$, M. MCB vs. M. HCB $P = 0.006$

in males than in females, and the increase in DCA/CA by probiotics was more prominent in females, although these changes did not reach statistical significance.

Several studies have suggested the pro-inflammatory effects of HFD by promoting low-grade intestinal inflammation in mice [8, 21] and by elevating pro-inflammatory cytokines by HFD [22]. One of the mechanisms is thought to be intestinal microbial changes and dysbiosis, which result in immunological dysregulation and inflammation in old age and are related to the tumorigenic effect in colorectal cancer [23, 24]. Meanwhile, there were sex-based differences according to sex in the response of inflammatory markers to probiotics. There have been studies on age- and sex-based differences in the occurrence and progression of intestinal inflammation [9, 20, 25–27]. First, two studies reported changes in gut microbiota due to aging in rats [20, 25]. Our team also reported a sex difference in the alteration of the gut microbiome by HFD [9], the occurrence of stress-induced intestinal inflammation [26], and the response to the administration of the probiotic *Lactobacillus farciminis* [27]. In these studies, female mice were more sensitive to stress than males [26, 27], while probiotics were more effective in females [27]. In addition, there were also results of sex differences in gut microbiota changes regarding intestinal inflammation in IL-10 knockout mice [28] and rat models [29]. These changes in gut microbiota according to age, sex, and diet are thought to cause changes in intestinal SCFAs such as butyric acid and acetic acid [30]. In the present study, HFD-induced intestinal inflammation did not show clear sex differences, but responses to Biovita or *C. butyricum* administration generally seemed to be more prominent in males than in females, although the results were inconsistent. These sex differences are thought to be due to the role of sex hormones, as the gut microbiome is involved in the excretion and circulation of sex hormones, which is called the microgenderome [31]. Estrogen directly controls the metabolism of bacteria through the combination with estrogen receptor beta, ERβ [32], and bacteria are also actively involved in the metabolism of estrogen [33]. That is, estrogen increases intestinal permeability through its activating effects on immune cells, and influences the composition of gut microbiota [34], although there are conflicting reports stating the effect of estrogen on chronic inflammation [35]. And the gut microbiota are not only influenced by estrogen, but also engage in the metabolism of estrogen [33], that β-glucuronidase in some taxa in the gut microbiota mutates the estrogen that flows into the bile [33]. An association between total estrogen levels in the urine and the richness and α-diversity of fecal bacteria was observed in males and menopausal females but not in pre-menopausal females [31,

36], that suggests non-ovarian estrogen is associated with gut microbiota and β-glucuronidase [36]. Sex hormones can cause differences in the incidence of intestinal diseases such as colorectal cancers [37], as it is well-known that it occurs more frequently in an aged male population, either young or female [38]. As an example of an association between intestinal inflammation and colon cancer, MPO, an inflammatory marker, was found to be linked to the severity of dextran sulfate sodium (DSS)-induced colitis [39] and was reported as an indicator of colon cancer risk [40].

Probiotics can change the composition and balance of the gut microbiota and inhibit micro-inflammation of the intestine through anti-inflammatory actions [10], probably through the production of SCFAs such as butyric acid. In the present study, we used Biovita and three different concentrations of *C. butyricum.* The Biovita 3 bacterial species complex consists of three probiotic bacterial strains, as mentioned above (*C. butyricum*, *L. sporogenes*, and *B. subtilis*), and the beneficial action of Biovita is mainly thought to be due to the production of butyric acid in *C. butyricum* [12]. As mentioned above, supplementation with the naturally occurring butyrate-producing probiotic *C. butyricum* CGMCC0313.1 reduced lipid accumulation in the liver and serum, lowered circulating insulin levels, and improved glucose tolerance and insulin sensitivity in an HFD-induced obese mouse model [11]. In addition, it reversed HFD-induced colitis, as shown by the reduction of TNF-α and increase of IL-10 and IL-22 levels in colon tissues, increased TJPs claudin-1 and occludin to reduce colon permeability, and increased intestinal SCFAs, including butyric acid and acetic acid [11]. In the human gut, *C. butyricum* has a fermentative lifestyle and can consume undigested dietary fibers and generate SCFAs, specifically butyrate and acetate. These beneficial effects are thought to be due to butyrate production. Butyrate is one of the dominant SCFAs produced by fermentation of dietary fiber by Firmicutes and *Clostridium* clusters [41] via the butyrate kinase pathway [42]. It is the preferred energy source for the colonic mucosa and is known to have anti-inflammatory and anti-proliferative effects, induce cell differentiation, promote cell apoptosis, and reduce tumor cell invasiveness [43, 44]. Furthermore, it is known to suppress the growth of tumor cells via inhibition of histone deacetylases and inactivation of many oncogenic signaling pathways [45, 46]. So, butyrate is thought to be able to improve inflammation through these mechanisms. Although the anti-inflammatory effects were not clearly and consistently derived in our data, it would be possibility due to small number of subjects or the insufficient concentration of probiotics.

The effect of BAs on the gut microbiota has recently been reported [47]. BAs regulate mucosal homeostasis and inflammation [13], via direct interactions with both germline-encoded cellular receptors and luminal bacteria [14]. BAs shape microbial colonization in the gut due to intrinsic bacteriostatic activities [48] but are also metabolized by many commensal bacteria in the intestinal lumen [49, 50]. Therefore, it can be assumed that when the composition of intestinal microbial changes during intestinal inflammation, a change in bile composition occurs, altering intestinal inflammation. A previous study showed that serum and hepatic levels of CA tended to decrease, whereas serum and hepatic DCA levels did not change in HFD-fed control rats [51]. In contrast, the concentrations of fecal secondary BAs, such as DCA, were significantly lower, whereas the concentrations of primary BAs, such as CA, were significantly higher in patients with ulcerative colitis (UC) than in healthy controls in humans [52]. The decrease in secondary BAs compared to that of primary BAs could be due to impaired BA metabolism followed by gut dysbiosis. Therefore, we tried to measure the fecal levels of primary and secondary BAs and assumed that a decrease in DCA/CA, that is, a further decrease in secondary BA (DCA) compared to primary BAs (CDCA, CA) could be interpreted as gut microbial dysbiosis. DCA/CA decreased in the HFD groups compared to the control groups in both males and females, although the difference was not statistically significant. DCA/CA seemed to increase in high-concentration *C. butyricum* group in males and in low and high-concentration *C. butyricum* groups in females that variations of overall BA levels were larger in males, but the increase of DCA/CA by probiotics seemed to be more prominent in females. This heterogeneity could be due to the small sample size, but it could be an indicator of sex differences in gut dysbiosis due to the effects of sex hormones, as steroid sex hormones and BAs have a structural similarity because they are both derivatives of cholesterol, which contains the cyclic steroid nucleus [53]. Furthermore, they can be recycled through the enterohepatic circulation process, which is in part regulated by the gut microbiota. Therefore, further studies on the correlation between BAs, gut microbiota, and sex hormones are required.

A defective intestinal epithelial tight junction barrier has been shown to be a pathogenic factor in the development of intestinal inflammation [15]. In this study, sex differences were observed in the change in the expression of TJPs according to the HFD and probiotics. Although not a study on HFD-associated intestinal inflammation, our team previously observed differences according to disease subtype and sex in the expression of TJPs in patients with IBS; female patients with IBS showed significantly lower ZO1 mRNA expression levels, especially in diarrhea and mixed-type IBS than control females [54]. In our knowledge, there was no study showed clear relationship between fat deposition, TJPs and sex, but there are several studies could explain the mechanisms. First, a study demonstrated the role of gonadal white adipose tissue lipid storage and lipolytic metabolites in

inducing inflammation in males and remodeling in females [55]. They concluded that fat depot distribution and lipolysis are more likely to be tightly regulated in females than in males to promote lipid and glucose homeostasis and prevent metabolic syndrome in reproductive years under the influence of sex hormones. Another study reported that females with metabolic syndrome showed a lower concentration of the anti-inflammatory adiponectin, whereas males showed increased levels of pro-inflammatory markers. That is, there are sex-associated differences in the pattern of obesity-induced inflammation, and excessive production of proinflammatory cytokines is more prominent in males, while reduced levels of the anti-inflammatory adipokine adiponectin are observed in females [56].

Metabolic stresses derived from nutrient overload are known to cause inflammations, that the excess energy is converted to fat and results in accumulation of fat in the adipocytes, and to increase intestinal cell membrane permeability, possibly through changes in gut microbiota composition followed by immune cell recruitment [57]. And sex hormone estrogen mediates sex-dependent differences in energy metabolism by regulating lipogenesis, lipolysis and the location of fat deposition [58]. Since we did not analyze the difference in metabolic pathways according to sex in this study, so we cannot conclude based on our results. But sex-dependent inflammations and responses of inflammation according to the administration of probiotics observed might be caused by these mechanisms, under the influence of sex hormones and associated gut microbial changes. So, research on sex differences in the metabolic and molecular aspects in intestinal inflammation would be needed.

Our study had several limitations. First, changes following HFD and probiotics administration were not consistent thoroughly and somewhat heterogenous, that changes in the expression of inflammatory markers in males and changes in the expression of TJPs in females were statistically significant only in some parts, and relative differences in changes compared to the opposite sex were shown in general. However, although not statistically significant in all cases, there was a consistent trend of different response patterns to HFD and probiotics between the sexes. In addition, the reason that the decrease in inflammatory markers by probiotics was not noticeable may be that the intervention effect of probiotic agents was not strong because Biovita is a health functional product, not a medical drug, and the concentration of *C. butyricum* in each group was determined based on the composition of Biovita. Alternatively, this inconsistency may be due to the small sample size or the uncertainty of animal experiments; even if the experiment was conducted under the same conditions, it may show different results. Second, it is necessary to analyze gut microbial changes to logically explain the inflammatory response to diet. The rationale for this response needs to be clarified through fecal microbiome sequencing studies, so we conducted a next generation sequencing study. However, the experimental results are vast and have not yet been organized, and we plan to present them as a follow-up study soon. Third, the results were only from young rats; therefore, age differences could not be analyzed. Initially, we conducted the experiment in both old age (2-year-old) and young age (6-week old) groups of either sex. However, due to the difficulty of breeding (18 months from 6-month-old in our animal facility), there were not enough old rats available, and some of them even died during the gavage period. Therefore, we had to exclude old rats from the results since we were unable to analyze the results, including the control group.

Despite these limitations, our study has strengths; that is, we analyzed the effect of probiotics, Biovita, and different concentrations of *C. butyricum* on HFD-induced intestinal inflammation in many aspects. To our knowledge, this is the first trial to measure SCFAs, BAs, and TJPs together with inflammatory markers and histology in both male and female rats. We observed sex differences between males and females in patterns of intestinal inflammation and response to probiotics; that is, *C. butyricum*'s suppression of weight gain, decrease in inflammatory markers, and increase in SCFAs were mainly found in males, while changes in TJPs and BAs were more significant in females. Different patterns of change in SCFAs and BAs between males and females and the expression of inflammatory markers and TJPs would suggest sex differences in the background of intestinal inflammatory mechanisms. Strict intervention is very difficult and control of various factors affecting the intestinal microbial environment such as diet is almost impossible in human. Thus we conducted this experiment using rats. Through this experiment, the possible beneficial effects of probiotic *C. butyricum* were observed, so we believe that this could be the basis for future research on human subjects.

In conclusion, rat males and females showed microscopic and molecular differences in their responses to HFD and probiotics, and probiotic *C. butyricum* improved some indicators of HFD-induced colonic inflammation such as levels of inflammatory markers. In addition, the production of SCFAs increased and the expression of TJPs also enhanced. However, these results were tended to be more pronounced in male rats, showing sex difference, and these need to be confirmed in human.

Author's contribution Conceptualization and generator: NK. Data curation: SIC, NK. Formal analysis: YC, SIC. Funding acquisition: NK. Investigation: SIC, RHN, JYJ, NK. Methodology: SIC, RHN, JYJ, NK. Project administration: NK, Y-JS. Resources: HM, Y-RK. Supervision: NK, HYN, Y-JS. Writing—original draft: YC, SIC. Writing—review and editing: NK, CMS, DHL.

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Declarations

Conflict of interest The authors have no conflicts of interest relevant to this article to disclose.

Ethical approval This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of South Korea. The protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital (Permission No. BA1506-178/027-01).

Informed consent This study is exempt from consent, since it is an animal experiment.

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