Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

Influence of gut microbiome composition on effect variations of anti-cholesterol treatment among individual mice

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ARTICLE INFO

Article history:
Received 10 May 2023
Received in revised form 24 July 2023
Accepted 18 January 2024

Keywords:
Gut microbiome
Anti-cholesterol
Bile salt hydrolase (BSH) activity
Bile acid synthesis
Faecal microbiota transplantation

ABSTRACT

This study investigated if the variation in the effect of anti-cholesterol (AC) treatment on individual mice are related to gut microbiome composition. The bile salt hydrolase (BSH) activity of 23 commercial fermented milk products was examined to select a fermented milk product for AC treatment. Mice were fed to different diets for 6 weeks: high-fat (60% of total calories from fat; D1), high-dietary fibre (20% cellulose; D2), and low-fat (17.2% of total calories from fat; D3) diets to change their gut microbiomes. Subsequently, faecal microbiome was transplanted (FMT) into mice treated with high cholesterol diet contained 2% cholesterol, followed by AC or non-AC (sterile tap water, STW) treatments. Control groups with normal (NC) and high-cholesterol diets (PC) were prepared for both AC and STW treatment. All experimental groups were subjected to serum and liver cholesterol, cholesterol metabolism-related (CMR) gene expression, and intestinal microbiome analyses. D3-FMT mice showed the most significant enhancements in cholesterol ratio and decreased hepatic cholesterol levels with AC treatment. Moreover, upregulation of the *Cyp7a1* gene expression was observed in this group. Furthermore, the intestinal microbiome analysis indicated higher abundances of BSH-producing *Eubacterium*, *Bifidobacterium*, and *Parabacteroides* in the D3-FMT + AC group compare to others, potentially contributing to increased bile acid synthesis.

1. Introduction

Cholesterol, a precursor of steroid hormones, bile acids, and vitamin D, is important for maintaining cell membrane integrity[1]. Cholesterol is either obtained from dietary intake or synthesized endogenously within various cells in the body, including the liver, testicle, and adrenal glands[1]. Approximately 50% of synthesized cholesterol comes from the liver[2]. Excess cholesterol in the blood may cause hypercholesterolemia, a known risk factor for cardiovascular diseases (CVD)[3-5]. Among the different classes of lipoprotein-cholesterol complexes, elevated concentrations of low-density lipoprotein (LDL) cholesterol increase the risk of CVD[6]. Hypercholesterolemia may be attributed to a diet rich in saturated fats, obesity, genetic disorders (familial hypercholesterolemia), or diabetes[3].

Probiotics are living microorganisms, and they can improve the health of an individual[7]. Lactobacilli are well-known probiotics that improve human health and prevent various diseases[8-9]. Many previous studies have shown that lactobacilli and fermented dairy products have cholesterol-lowering effects in animal models[10-11] and humans[12-13]. According to Miremadi et al.[14], lactic acid bacteria (LAB) play a role in decreasing cholesterol, primarily through the generation of bile salt hydrolase (BSH). This enzyme deconjugates conjugated bile salts with either taurine or glycine between hepatic and intestinal circulation[14]. The deconjugated bile salts exhibit reduced solubility and are less efficiently reabsorbed from the intestinal lumen compared to conjugated bile acids, which leads to

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Peer review under responsibility of Tsinghua University Press.

SciOpen Publishing services by Tsinghua University Press

http://doi.org/10.26599/FSHW.2022.9250252

the excretion of free bile acids in faeces[15]. Thus, cholesterol is used for *de novo* synthesis of bile acids to make up the bile acid released in faeces, resulting in decreased cholesterol levels[16].

The human body hosts at least 10^{14} microorganism cells, which is more than body cells[17]. The human gastrointestinal (GI) tract contains 10 million genes associated with different microbial species, and all genes have some beneficial impact on host health[18-19] with potential effects on the homeostasis of the immune system. However, because of differences in diet, the microbiome is different among individuals[20]. The dominant bacterial species in the human GI tract belong to Bacteroidota (e.g., *Porphyromonas* and *Prevotella*), Firmicutes (e.g., *Ruminococcus*, *Clostridium*, and *Eubacterium*), and Actinomycetota (*Bifidobacterium*)[21]. Other bacteria, such as lactobacilli, streptococci, and *Escherichia coli*, are found in small numbers[21-22]. Alterations of intestinal microbiome have been linked to many diseases and conditions, including obesity and metabolic syndrome[23-24]. Probiotics, antibiotics, and, more recently, faecal microbiota transplantation (FMT) are currently being evaluated as therapeutic approaches targeting dysbiosis and manipulation of the intestinal microbiome[25]. The interaction between intestinal microbiota and commonly used non-antibiotic drugs is complex[26]. Specifically, the composition of the intestinal microbiome can be affected by these medications[26]. Conversely, the intestinal microbiome can also directly influence an individual's reaction to a specific drug or treatment through enzymatic modifications to the drug's structure, thereby modifying its bioavailability, bioactivity, or toxicity[27-28]. In previous studies, it was observed that when specific bacteria were administered with drug to animal models, there was an enhancement in immune responses[29] or an improvement in the efficacy of anticancer agents[30].

Therefore, the objective of this study was to evaluate the interaction between the composition of the gut microbiome and the effect of anti-cholesterol (AC) treatment in mice.

2. Methods

2.1 Selection of fermented milk for AC treatment

Commercial fermented milk (CFM 1-23) products known to possess cholesterol-lowering effects manufactured by 10 companies were purchased from online markets in Korea. The expiration dates of the products were checked, and the products were purchased within 1–2 days of manufacture.

2.1.1 Measurement of BSH activity

Fermented milk samples were examined for hydrolase activity against tauro- or glycol-deoxycholic acid (TDCA or GDCA) with the plate assay method[31]. Briefly, 100 μL of CFM samples were spread-plated on MRS agar (Becton, Dickinson and Company, Franklin, NJ, USA), and the plate was incubated at 37 °C for 24 h. MRS medium allows a more abundant growth of LAB, and the LAB in fermented milk are isolated easily in MRS medium[32]. Subsequently, isolated colonies on MRS agar were streaked on MRS agar supplemented with 9.6 mmol/L TDCA (Sigma, St. Louis, MO, USA; T0875) or 2 mmol/L GDCA (Sigma; G9910) and incubated anaerobically in a tight container containing an anaerobic pack (Anaerogen™; Oxoid, Hampshire, UK) at 37 °C for 48 h. MRS agar plates without the supplements were the control. The presence of precipitated bile acid around the colonies (opaque halo) was considered a positive reaction.

2.1.2 Analysis of cholesterol assimilation

Cholesterol assimilation was measured according to the method described by previous studies[33-34], with some modifications. 10 mg of cholesterol (Sigma) and 22 mg of *L*-*α*-lecithin (Sigma) were dissolved in 1 mL of chloroform (Duksan, Ansan, Gyeonggi, Korea), and chloroform was evaporated under nitrogen gas. Then, 10 mL of 0.4 mol/L sucrose (Samchun, Pyeongtaek, Korea) was added to the cholesterol/lecithin mixture and was sonicated (Bransonic Sonicator CPX-3800H; Branson Ultrasonics, Danbury, CT, USA) to produce cholesterol micelles. This micellar solution was centrifuged at 20 000 × *g* at 4 °C for 30 min (Combi 514R centrifuge; Hanil Science, Incheon, Korea), and the supernatant was then filtered through a 0.45-μm filter (Sartorius, Goettingen, Germany). 100 mL 1% fermented milk were placed into a mixture of 1 mL of the micellar solution and 9 mL MRS broth supplemented with 0.2% thioglycolic acid (Sigma) and 0.3% oxgall (Becton, Dickinson and Company). This resultant containing 91 μg/mL of cholesterol was incubated at 37 °C for 18 h. After the incubation, the culture solution was centrifuged at 7 000 × *g* and 4 °C for 10 min to remove the bacterial cells, and the residual cholesterol of the supernatant was determined with the *o*-phthalaldehyde method by Rudel and Morris[35]. Briefly, 0.5 mL of the supernatant was transferred to a test tube. Subsequently, 3 mL of 97% ethanol and 2 mL of 50% KOH were added to the tube. The mixture was then heated at 60 °C for 10 min in a water bath. The samples were cooled, and 5 mL of hexane was then added to the tube. After vortexing the tube for 30 s, the tube was left at room temperature (20–25 °C) for 15 min and centrifuged at 7 000 × *g* for 10 min at 4 °C for phase separation. The hexane layer (2.5 mL) was transferred into a test tube and evaporated at 60 °C under nitrogen gas. After drying, 4 mL of *o*-phthalaldehyde reagent (Sigma) was added to the tube, and it was allowed to be left at room temperature for 10 min. For colour development, 2 mL of sulfuric acid was added to the tube, vortexed for 1 min, and incubated at room temperature for 10 min. The absorbance of this mixture was measured at 550 nm with a spectrophotometer (OPTIZEN 2120UV spectrophotometer; Mecasys, Daejeon, Korea) to quantify residual cholesterol. A standard curve was prepared with the standard: 0, 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL cholesterol in MRS broth (R^2 = 0.994). Cholesterol assimilated by the fermented milk samples was determined using the following equation[36]:

$$\text{Cholesterol assimilation (\%)} = \left(1 - \frac{A}{B}\right) \times 100 \quad (1)$$

Where A is the cholesterol content in the supernatant of a sample, and B is the cholesterol content of the control.

2.2 Animal model

The animal experiment was approved by the Institutional Animal

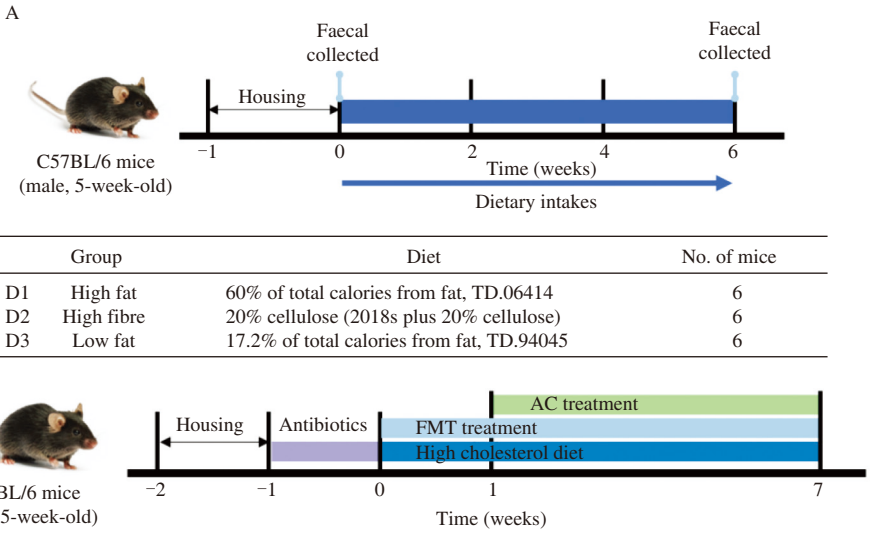
Care and Use Committee of Sookmyung Women's University (ethic review No. SMWU-IACUC-1905-012-02). Five-week-old male C57BL/6 mice, purchased from Raon Bio (Yongin, Gyeonggi, Korea), were housed in metal cages (3–4 mice/cage) at a controlled temperature ((21 ± 1) °C) and humidity (50%–60%) under a 12 h light/dark cycle. All mice were weighed at the beginning and allocated to one of the 10 groups to have similar mean weights.

2.2.1 *Preparation of gut microbiome inoculum*

To induce different gut microbiome compositions in mice, they were fed with high-fat diet (60% of total calories from fat; Envigo Teklad Custom Diet TD.06414, Wisconsin, USA; D1), 20% dietary fibre diet (2018S supplemented with 20% cellulose (Envigo Teklad Custom Diet); D2), and low-fat diet (17.2% of total calories from fat; Envigo Teklad Custom Diet TD94045, Wisconsin, USA; D3) customised by Doo Yeol Biotech (Seoul, Korea) for 6 weeks (Fig. 1A). After the 6-week diet, a fresh faecal sample (200 mg) from the mice (donor) of each diet group was collected in the morning before every FMT treatment. The collected faecal sample was suspended in 5 mL PBS, vortexed for 3 min, and settled by gravity for 3 min. The supernatant was used as the microbiome inocula for FMT. The collected faecal samples were also used for microbiome analysis, thus, stored at −80 °C until DNA extraction. For the faecal sample, a comparative analysis of NGS amplicon sequencing was performed by amplifying the V3–V4 regions of the 16S rRNA. Library preparation and paired-end sequencing were performed in Macrogen (Korea) using the MiSeq™ platform (Illumina, San Diego, CA, USA). The sequencing reads were trimmed and filtered in each sample dataset based on sequence length and quality score (Q = 15) with CLC Genomics workbench v20.0.4 (CLC Bio, Denmark). The SILVA ribosomal RNA database (Bremen, Germany) was used for operational taxonomic units (OTUs) analysis to obtain taxonomic information. The major sequence of each OTU was referred to the NCBI 16S rRNA database, and taxonomic information was obtained using nucleotide blast databases (BLASTn). Subsequently, the faecal microbiome compositions were compared among the diet treatments.

2.2.2 *Induction of different microbiome compositions in intestine and AC treatment*

During the first week of acclimation, mice received sterile standard chow and water *ad libitum*. To deplete the gut commensal microflora in mice, they were treated with antibiotics (vancomycin (for Gram-positive bacteria; 500 mg/L; Aladdin®, Shanghai, China), metronidazole (for anaerobic bacteria and some protozoa; 1 g/L; Aladdin), and ciprofloxacin (for Gram-negative bacteria; 200 mg/L; Aladdin), which can cover a broad spectrum of bacterial species[37], mixed in their drinking water for 5 days. To confirm changes in the intestinal microbiota, the faecal samples obtained before and after antibiotic treatments were plated on Brain Heart Infusion agar (BHI



	Group	Diet	No. of mice
D1	High fat	60% of total calories from fat, TD.06414	6
D2	High fibre	20% cellulose (2018s plus 20% cellulose)	6
D3	Low fat	17.2% of total calories from fat, TD.94045	6

Group		Diet	No. of mice
AC treatment group	NC + AC	Normal diet + AC	10
	PC + AC	High cholesterol diet + AC	10
	D1-FMT + AC	High cholesterol diet + D1 + AC	10
	D2-FMT + AC	High cholesterol diet + D2 + AC	10
	D3-FMT + AC	High cholesterol diet + D3 + AC	10
STW treatment group	NC + STW	Normal diet + STW	10
	PC + STW	High cholesterol diet + STW	10
	D1-FMT + STW	High cholesterol diet + D1 + STW	10
	D2-FMT + STW	High cholesterol diet + D2 + STW	10
	D3-FMT + STW	High cholesterol diet + D3 + STW	10

Fig. 1 Experimental design overview. Study design to induce variations in gut microbiota compositions through diet intake (A) and to investigate the impact of gut microbiome on the anti-cholesterol effect of AC treatment (B). NC, negative control; PC, positive control; D1-FMT, FMT for high-fat diet mice faecal; D2-FMT, FMT for high-dietary fibre diet mice faecal; D3-FMT, FMT for low-fat diet mice faecal.

agar; Becton, Dickinson and Company; Franklin, NJ, USA) and cultured under anaerobic conditions. To allow the colonisation of different microbiome compositions in the intestine of mice, they were administered with a normal diet (NC; 2018S, Envigo, Indianapolis, IN, USA), high-cholesterol diet (2%) (PC; TD.01383; Envigo), and high-cholesterol diet with FMT (D1-FMT, D2-FMT, and D3-FMT). FMT was conducted by oral administration of the microbiome inocula (200 μL; D1, D2, and D3) for 7 weeks (Fig. 1B). The mice were fed a normal diet and high-cholesterol diet *ad libitum*, and FMT was treated in mice twice a week. One week after the FMT started, AC or sterile tap water (STW) treatments (10 mL/kg of body weight) were administered by oral gavage to the mice every 2 days for 6 weeks (Fig. 1B). For AC treatment, CFM-2, which showed the highest AC effect, was used. Thus, treatment groups were 1) NC + AC, 2) PC + AC, 3) D1-FMT + AC, 4) D2-FMT + AC, 5) D3-FMT + AC, 6) NC + STW, 7) PC + STW, 8) D1-FMT + STW, 9) D2-FMT + STW, and 10) D3-FMT + STW. All mice were weighed at the beginning and randomly allocated to the treatment group (Fig. 1B). The mice were fasted for 24 h before euthanization. The mice were euthanised via anesthetisation with isoflurane (Hana Pharm Co., Ltd., Hwaseong, Korea).

2.3 Measurement of weight gain and food efficiency

Food intake (g) and weight (g) were measured each week during treatment. The cholesterol consumption (mg/day) of each group was calculated from food intake. Weight gain ratio (%) and food efficiency (%) were calculated using the following equations:

$$\text{Weight gain ratio (\%)} = \frac{m_{7w} - m_{0w}}{m_{0w}} \times 100 \quad (2)$$

Where m_{7w} is the weight (g) at 7th week, and m_{0w} is the weight (g) before treatment.

$$\text{Food efficiency (\%)} = \frac{\text{Weight gain (g/day)}}{\text{Food intake (g/day)}} \times 100 \quad (3)$$

The weights of the liver and white adipose tissue (WAT) were measured, and the liver/body weight and WAT/body weight ratios were calculated.

2.4 Analysis of gut microbiome composition after AC treatment

The gut microbiome of mice (n = 3) in each treatment group was analysed to examine the changes in the microbiome compositions induced by FMT and AC. Caecum faecal samples were collected after euthanization, and DNA was extracted with the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. According to the manufacturer's instructions, faecal DNA samples were a comparative analysis of NGS amplicon sequencing with an Illumina MiSeq sequencing system. Sequencing libraries were prepared according to Illumina 16S rRNA sequencing library protocols, and the V3–V4 regions of DNA were amplified. Paired-end sequencing was performed with the Illumina MiSeq system. The SILVA ribosomal RNA database was used for the OTU analysis. The major sequence of each OTU was referred to the NCBI 16S rRNA database, and taxonomic information was obtained using BLASTn. Among the gut microbiome compositions that show differences among groups, the relative abundances of specific microbiome that affected cholesterol metabolism were analysed.

2.5 Analysis of serum and liver

Blood was collected from the posterior aorta of mice after euthanization with a syringe and stored in a serum-separating tube (Microtainer; Becton and Dickinson, Franklin Lakes, NJ, USA) for 30 min. Thirty milligrams of mouse liver were placed on ice to avoid RNA degradation. The sample was placed in a 2-mL microcentrifuge tube containing one stainless steel bead (5-mm diameter, Qiagen) on ice. The sample was homogenized by a tissue lyser (TissuLyser LT, Qiagen) at 50 Hz.

2.5.1 Serum

Blood samples were centrifuged at 2 340 × g and 4 °C for 10 min, and the supernatant (serum) was transferred into a sterile microcentrifuge tube and stored at −70 °C until used. Total cholesterol (TC), HDL-cholesterol (HDL-c), and LDL-cholesterol (LDL-c) concentrations in the serum were determined with the BS-200E Chemistry Analyzer (Mindray Medical International Ltd., Shenzhen, China).

2.5.2 Liver

TC contents in the liver were measured using a TC assay kit (AM 202-K, Asan Pharm Co., Seoul, Korea) containing cholesterol esterase and oxidase at a wavelength of 500 nm with a spectrophotometer (Take3; BioTek Instruments, Inc., Winooski, VT, USA). Lipids from the liver were extracted with a 2:1 (*V*/*V*) chloroform-methanol mixture. Aliquots of extracts containing cholesterol were transferred to screw-cap tubes and dried under nitrogen gas. Because complete removal of the solvent was necessary to avoid interference in the assay, the tube was heated for 30 min in a nitrogen gas evaporator at 80 °C. Subsequently, 1 mL of ethanol was added to dissolve the sample in the tube. Thereafter, 10 μL of the sample solution was mixed with 1.5 mL of an enzymatic reagent containing cholesterol esterase and cholesterol oxidase and allowed to stand at 37 °C for 5 min. The absorbance of this mixture was measured at 500 nm. For the blank, the enzymatic reagent was used.

2.5.3 Quantification of gene expression in the liver

Liver mRNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To quantify gene expression, complementary DNA (cDNA) was synthesised from total mRNA with the QuantiTech Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. To quantify mRNA expression, quantitative reverse transcription PCR was performed with the QuantiTect SYBR® Green PCR kit (Qiagen) and Rotor-Gene Q (Qiagen). The mRNA expression of genes related to cholesterol metabolism listed in Table 1 was analysed and normalized with the expression level of mouse *Gapdh* as an endogenous control gene. The primer sequences used are listed in Table 1. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method[38].

Table 1
Primer sequences of cholesterol metabolism-related genes used for quantitative reverse transcription PCR.

Gene	Protein name		Primer sequence	Reference
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Forward	5′-CCA CAG CTG AGA GGG AAA TC-3′	[87]
		Reverse	5′-AAG GAA GGC TGG AAA AGA GC-3′	
Nr1h3	Nuclear receptor 1H3 (LXRα)	Forward	5′-CTC AAT GCC TGA TGT TTC TCC T-3′	
		Reverse	5′-TCC AAC CCT ATC CCT AAA GCA A-3′	
Nr1i2	Nuclear receptor 1I2, pregnane X receptor (PXR)	Forward	5′-TAG GGA CCT GCC TAT TGA GGA-3′	
		Reverse	5′-CCG TTT CCG TGT CGA ACA TC-3′	
Abcg5	ATP binding cassette subfamily G member 5	Forward	5′-TGA TTG GCA GCT ATA ATT TTG GG-3′	[88]
		Reverse	5′-GTT GGG CTG CGA TGG AAA-3′	
Abcg8	ATP binding cassette subfamily G member 8	Forward	5′-TGC TGG CCA TCA TAG GGA G-3′	
		Reverse	5′-TCC TGA TTT CAT CTT GCC ACC-3′	
Cyp7a1	Cytochrome P450 7a1, cholesterol-7-alpha hydroxylase	Forward	5′-ACG GGT TGA TTC CAT ACC TGG G-3′	[89]
		Reverse	5′-TGT GTC CAA ATG CCT TCG CAG-3′	
Ldlr	LDL receptor	Forward	5′-CAT CAA GGA GTG CAA GAC CAA CG-3′	
		Reverse	5′-CAC TTG TAG CTG CCT TCC AGG TTC-3′	

2.6 Statistical analysis

All the results were expressed as mean ± standard deviation values. The data were analysed using one-way ANOVA with the general linear model procedure of SAS® version 9.4 (SAS Institute Inc., Cary, NC, USA). Significant differences were compared with Tukey's HSD among groups at $\alpha = 0.05$.

3. Results and discussions

3.1 Donor microbiome for faecal microbiota transplantation

In this study, the microbiota was transplanted with faeces induced by high-fat, low-fat, and high-fibre diets. High-fibre diets, which is considered a positive-treatment group, is known as effectively reducing serum cholesterol concentrations by inhibiting cholesterol absorption[39]. The ratio of Firmicutes to Bacteroidota in the gut microbiome has been widely accepted as a marker of intestinal homeostasis[40]. Previous research has shown that an imbalance in this ratio is associated with various diseases such as obesity, type 2 diabetes, and cardiovascular disease[41]. Therefore, this study chose to manipulate this ratio through donor mice fed different diets to investigate its potential impact on the effects of AC treatment. Before the mice were fed a high-fat diet (D1), a high dietary fibre diet (D2), and a low-fat diet (D3), the ratio of Firmicutes to Bacteroidota in the intestine was approximately 1:1 (Fig. 2A). After 6-week treatment, the ratio changed to 3:1 in D1 and 1:3 in D2 mice, but the ratio of the D3 mice remained unchanged (Fig. 2B). Additionally, Actinomycetota was present with a small proportion (0.11%) only in the gut microbiome of D3 mice but not in D1 and D2 mice (Fig. 2B). At the family level, relative abundances of Lachnospiraceae and Ruminococcaceae OTUs belonging to Firmicutes were 21% and 20%, 5.9% and 5.9%, and 15% and 5.9% in D1, D2, and D3 mice, respectively (Fig. 2C). Meanwhile, Bacteroidaceae and Muribaculaceae were the predominant families belonging to Bacteroidota (Fig. 2C). The relative abundances of Bacteroidaceae were 2.1%, 24%, and 7% in D1, D2, and D3 mice, respectively, and those of Muribaculaceae were 11.6%, 13.6% and 17.3% in D1, D2, and D3 mice, respectively (Fig. 2C). In particular, Bifidobacteriaceae belonging to Actinomycetota had a relative abundance of 0.09% of the total gut microbiota of D3 mice (Fig. 2C).

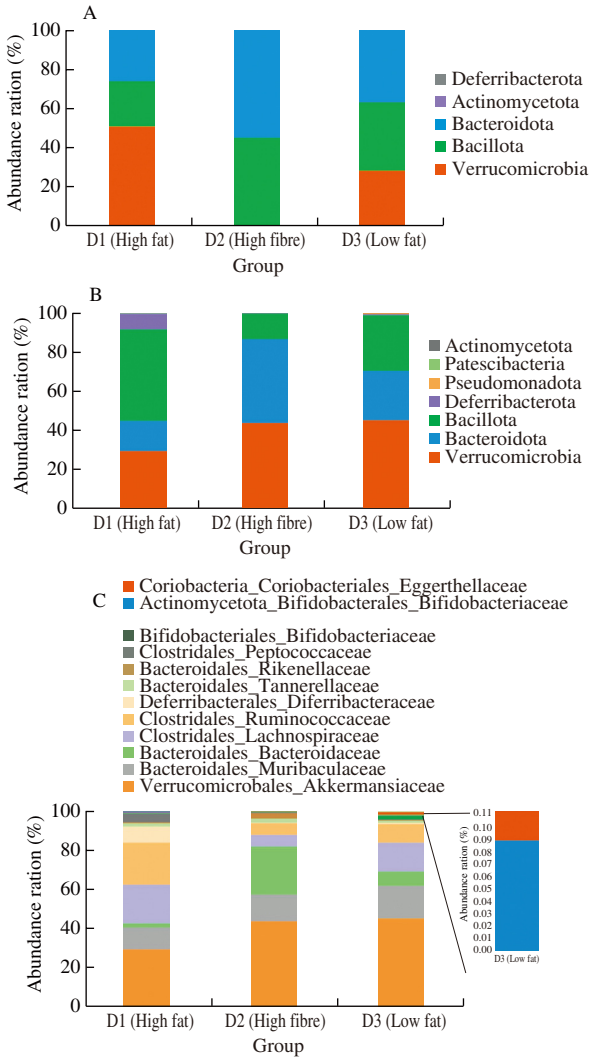


Fig. 2 OTU analysis of donor mice gut microbiota. (A) The phylum-level relative abundance of the donor's faecal microbiome at the first time point (0 week). The relative abundance of the donor's faecal microbiome at the phylum (B) and family (C) taxonomic levels after a period of 6 weeks. The data is based on the 16S rRNA gene sequences.

3.2 Selection of fermented milk for AC treatment

In a qualitative direct plate assay, BSH positive is indicated by either white colonies with surrounding precipitation zones or opaque white colonies without precipitation zones. TDCA or GDCA activity is a well-recognized indicator of BSH activity in bacteria[42]. Thus, the TDCA and GDCA activities of 23 CFMs were examined. 14 fermented milk products showed BSH activity positive against TDCA, and CFM-2, CFM-8, and CFM-16 showed BSH activity positive against both TDCA and GDCA (Table 2). The 14 CFMs were subjected to the analysis of cholesterol assimilation, and a cholesterol assimilation rate of more than 30% was considered a significant reduction rate[43-44]. Thus, 6 CFMs had more than 30%, and CFM-2 showed the highest cholesterol assimilation (49.9%), followed by CFM-11 (36.5%), CFM-5 (36.4%), CFM-13 (35.1%), CFM-8 (34.1%), and CFM-6 (30.9%) (Fig. 3). These results indicate that CFM-2 has higher AC effects with the highest BSH activity and cholesterol assimilation than the other CFMs. Therefore, CFM-2 was selected as an AC treatment for the animal experiments.

Table 2
TDCA and GDCA activity of the fermented milk products.

Sample	Plate assay activity	
	TDCA	GDCA
CFM-1	++ (p)	−
CFM-2	++ (p)	+ (p)
CFM-3	−/+ (w)	−
CFM-4	+ (p)	−
CFM-5	++ (p)	−
CFM-6	+ (p)	−
CFM-7	+ (p)	−
CFM-8	+ (p)	+ (p)
CFM-9	−	−
CFM-10	−	−/+ (w)
CFM-11	++ (p)	−
CFM-12	−/+ (w)	−
CFM-13	+ (p)	−
CFM-14	+ (p)	−
CFM-15	+ (p)	−
CFM-16	++ (p)	+ (p)
CFM-17	+ (p)	−
CFM-18	−	−
CFM-19	++ (p)	−
CFM-20	−	−
CFM-21	−	−/+ (w)
CFM-22	−/+ (w)	−
CFM-23	−	−

Note: −, no BSH activity; ++ (p), high BSH activity with precipitation; + (p), positive BSH activity and formation of opaque white colonies; −/+ (w), weak BSH activity and production of opaque white colonies.

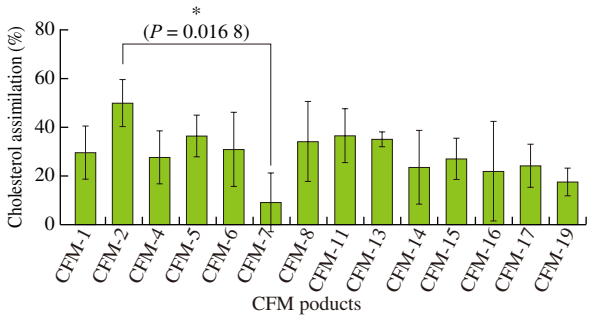


Fig. 3 Cholesterol assimilation of CFM products. Statistically significant differences among 14 product samples are indicated by asterisks (*$P < 0.05$).

3.3 Different effects of AC treatment among individual mice

3.3.1 Weight of organs and feed efficiency

During the FMT and AC treatments, the body weights of all mice in each group increased (Table 3), but there was an initial decrease in body weights during the first week due to antibiotic treatments, along with a depletion (98.7%, about −1.9 lg(CFU/g)) in gut microbiota (Fig. 4). No difference in the daily feed intake and the feed efficiency ratio were observed among the high cholesterol-induced groups (Table 3). It indicates that similar amounts of high-cholesterol diets were provided to the mice to induce high cholesterol. The liver/body weight ratios of mice were lower ($P < 0.05$) in the NC and D3-FMT groups than those in the other high cholesterol groups (Table 3). Previous study reported that a significant increase of the liver weight was observed in the high cholesterol diet groups, compared to the low cholesterol diet group[45]. Thus, it indicates that D3-FMT induces a lower liver weight in mice compared to the other FMT groups.

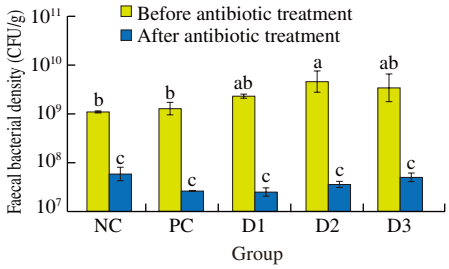


Fig. 4 Depletion of gut microbiota after antibiotic treatment. Faecal bacterial loads in all groups of mice. [a-c] Different letters indicate significant difference ($P < 0.05$).

Table 3
Body weight, body weight gain, food intake, and food efficiency of mice fed experimental diet for 7 weeks.

Group		Weight gain (g/day)	Food intake (g/day)	Food efficiency ratio (%)	Liver weight/body weight	WAT weight/body weight
AC treatment group	NC + AC	0.11 ± 0.03^{a}	1.03 ± 0.24^{b}	10.9 ± 2.7^{a}	0.041 ± 0.003^{de}	0.041 ± 0.014^{a}
	PC + AC	0.10 ± 0.02^{abc}	1.19 ± 0.16^{a}	8.3 ± 1.6^{b}	0.046 ± 0.003^{ab}	0.038 ± 0.007^{abc}
	D1-FMT + AC	0.10 ± 0.02^{abc}	1.15 ± 0.16^{a}	8.4 ± 1.4^{b}	0.044 ± 0.003^{bc}	0.031 ± 0.005^{cd}
	D2-FMT + AC	0.09 ± 0.01^{abc}	1.19 ± 0.17^{a}	7.9 ± 1.0^{b}	0.046 ± 0.002^{ab}	0.032 ± 0.007^{cd}
	D3-FMT + AC	0.10 ± 0.03^{abc}	1.12 ± 0.16^{ab}	8.7 ± 2.4^{b}	0.043 ± 0.002^{cd}	0.034 ± 0.005^{bcd}
STW treatment group	NC + STW	0.09 ± 0.02^{bc}	1.01 ± 0.10^{b}	8.9 ± 1.9^{b}	0.041 ± 0.002^{e}	0.027 ± 0.007^{d}
	PC + STW	0.11 ± 0.02^{ab}	1.19 ± 0.17^{a}	8.9 ± 1.4^{b}	0.048 ± 0.002^{a}	0.037 ± 0.008^{abc}
	D1-FMT + STW	0.10 ± 0.02^{abc}	1.21 ± 0.16^{a}	8.3 ± 1.6^{b}	0.047 ± 0.003^{a}	0.041 ± 0.008^{ab}
	D2-FMT + STW	0.09 ± 0.02^{c}	1.17 ± 0.16^{a}	7.5 ± 2.0^{b}	0.045 ± 0.003^{bc}	0.042 ± 0.007^{a}
	D3-FMT + STW	0.09 ± 0.02^{bc}	1.16 ± 0.18^{a}	8.1 ± 1.4^{b}	0.043 ± 0.001^{cde}	0.041 ± 0.006^{a}

Note: [a-d] different letters indicate significant difference in same column ($P < 0.05$).

3.3.2 Changes in gut microbiome

Throughout the duration of the antibiotic treatment, a significant reduction (98.7%) in the density of faecal bacteria was observed in the treated mice as compared to the untreated mice (Fig. 4). One week after FMT treatment, all mice had a recovery in faecal bacterial density, indicating partial recolonization of the intestinal microbiota due to FMT treatment. After 7 weeks with the FMT and AC treatments, the abundance of Erysipelotrichaceae belonging to Firmicutes was higher in the FMT + AC groups compared to the FMT + STW and PC groups (Fig. 5A). Among Erysipelotrichaceae, *Faecalibaculum rodentium* produced short-chain fatty acids that contributed to control the cholesterol contents in the body[46]. The abundances *F. rodentium* were 0.53%, 0.50%, and 0.34% of the microbiome for the D1-FMT + AC, D2-FMT + AC, and D3-FMT + AC groups, respectively. AC treatment changed the microbiome composition which may control the cholesterol contents. In addition, the abundance of Ruminococcaceae, belonging to the phylum Firmicutes, showed a higher in the D2-FMT and D3-FMT groups compared to the other high cholesterol groups (Fig. 5A). According to the previous study[47], the abundance of Ruminococcaceae was found to have beneficial effects on health and production of short-chain fatty acids, and it was observed to be decreased in obese women. One of the species belonging to Ruminococcaceae, the abundances *Ruminococcus champanellensis* were 1.48% and 1.23% of the microbiome for the D3-FMT + AC and D3-FMT + STW groups, respectively, which were higher than other experimental groups (Fig. 5B). *R. champanellensis* had positive correlation with plasma cortisol levels[48], and cortisol may increase cholesterol synthesis and circulation, potentially leading to elevated hepatic cholesterol contents[49]. Among Firmicutes, the genus *Eubacterium* accounted for 1.7%, 1.2%, and 1.0% of the microbiome of the D2-FMT + STW, D3-FMT + STW, and D3-FMT + AC groups, respectively (Fig. 5A). Especially, *Eubacterium coprostanoligenes* accounted for 1.7%, 1.0%, and 1.0% of the microbiome of the D2-FMT + STW, D3-FMT + STW, and D3-FMT + AC groups, respectively (Fig. 5C). *E. coprostanoligenes* may be a cholesterol-reducing species[50]. In addition, *E. coprostanoligenes* may convert cholesterol to coprostanol as its end-product via an indirect pathway in the gut[50]. Since coprostanol is poorly absorbed in the intestinal and excreted as a neutral sterol in faeces, *E. coprostanoligenes* may help lower serum cholesterol by converting cholesterol to coprostanol[51]. The abundance of Muribaculaceae, which family levels belong to Bacteroidoda, was increased in the D2-FMT (50.5%) and D3-FMT (35.8%) groups by AC treatment (Fig. 5A). In the D3-FMT groups, the species *Parabacteroides merdae* was present in higher proportions in the D3-FMT + AC (0.9%) and D3-FMT + STW (1.7%) groups than in the other groups (Fig. 5D). *Parabacteroides* was reported

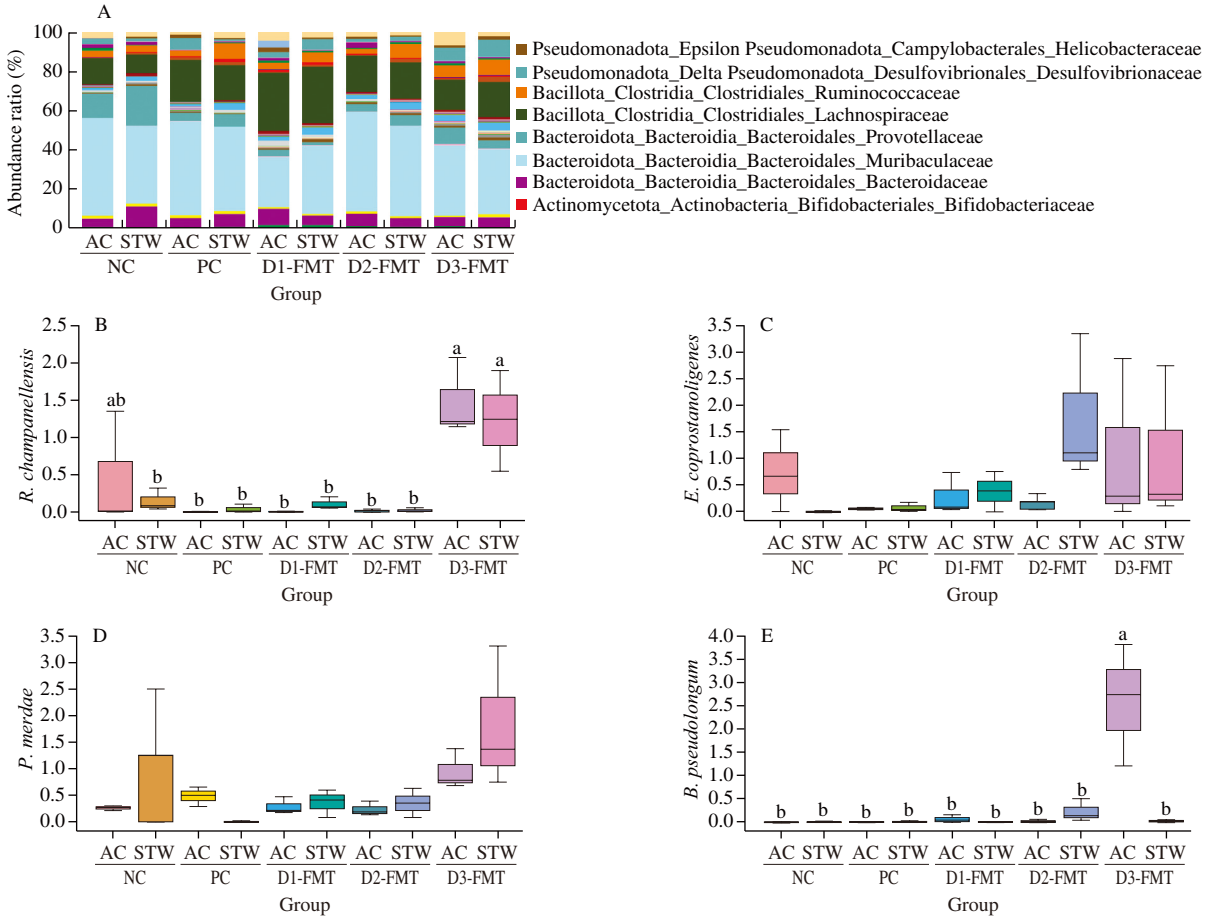


Fig. 5 Bacterial taxonomic compositions in gut. (A) The family-level relative abundance of the mice gut microbiota following FMT and AC treatments for 7 weeks. Boxplots illustrating the taxonomic abundance of certain gut microbiota in response to FMT and AC treatments in mice with high cholesterol (B) *R. champanellensis*, (C) *E. coprostanoligenes*, (D) *P. merdae*, and (E) *B. pseudolongum*. [a-b] Different letters indicate significant difference ($P < 0.05$).

to alleviate obesity and metabolic disorders[52]. A previous study reported that *Parabacteroides* reduced weight gain, improved glucose homeostasis, and corrected obesity-related abnormalities, including hyperlipidaemia and hepatic steatosis[53]. Especially, *P. merdae* effectively protected against cardiovascular damage in obesity mice model by enhancing branched-chain amino acid metabolism[54]. The proportion of Actinomycetota was significantly higher ($P < 0.05$) in the D3-FMT + AC (1.08%) group than in the other groups (Fig. 5A). It was also observed in D3-FMT donor mice, as presented in Fig. 2C. Among Actinomycetota, the species *Bifidobacterium pseudolongum* was present only in the D3-FMT + AC group (0.3%) (Fig. 5E). *B. pseudolongum* is a probiotic that has been shown to reduce body weight and cholesterol concentrations in mice[55] and human[56]. In addition, it has BSH, which might de-conjugate bile acid in the intestine[57]. Thus, the gut microbiome composition of D3-FMT compared to the other FMTs indicated the presence of certain bacterial species that showed a beneficial effect on reducing cholesterol levels in mice.

3.3.3 Cholesterol concentrations in serum

In HDL-c/TC ratio, the high cholesterol diet induced a decrease in HDL-c/TC ratio in mice, while the AC treatment increased HDL-c/TC ratios. The HDL-c/TC ratios were higher ($P < 0.05$) in D1-FMT + AC and D3-FMT + AC groups than those in D1-FMT + STW and D3-FMT + STW groups. Furthermore, this ratio of the D3-FMT + AC group was higher ($P < 0.05$) than that of the PC + AC group (Fig. 6A). Among the FMT + AC groups, the D3-FMT + AC group tended to have high HDL-c/TC ratio compared to the D1-FMT + AC and D2-FMT + AC groups. These results suggest that AC treatment generally increased HDL-c/TC ratios, and the most significant effect was observed in the D3-FMT + AC group. Even though, there were no obvious differences in the LDL-c/TC ratios between AC and STW treatments, the LDL-c/TC ratio in the D3-FMT + AC group tended to be lower than in the D3-FMT + STW group (Fig. 6B). These results suggest that the microbiome composition transplanted by D3-FMT might affect the effect of AC treatment. The D2-FMT groups had generally higher HDL-c/TC ratio and lower LDL-c/TC ratio than other experimental groups, but no significant change with AC treatment when compared to the D1- and D3-FMT groups (Fig. 6). It might be caused by the effects of the microbiome transplanted by D2-FMT and the presence of short-chain fatty acid-producing bacteria in the D2-FMT group which was derived from donor mice consumed a high-fibre diet[57-60]. FMT can change the gut microbiota and the metabolites in recipient[61]. Notably, a study by Hartley et al.[59] suggested that a high-fibre diet increases the colonies of short-fatty acid-producing bacteria in the intestines.

3.3.4 Cholesterol contents in liver

Compared with humans, rodents have higher rates of cholesterol and bile acids synthesis in the liver, along with faster clearance and lower concentrations of serum LDL-c[62]. LDL-c and HDL-c (especially in mice) in the serum are transported to the liver, where it is used as a precursor for synthesizing bile acids, leading to a reduction in hepatic cholesterol[62-63]. Most bile acid is then conjugated with the glycine or taurine in the liver, and the conjugated bile acid is transferred to the intestine[64]. However, if the bile acid is deconjugated by BSH of AC treatment, the deconjugated bile acid is excreted by faeces rather than being reabsorbed[65]. Eventually, it results in reduced cholesterol levels in the liver[66]. Thus, to investigate an effect of the FMT and AC treatments in the hepatic cholesterol contents, the cholesterol contents in the liver were analysed. The AC groups had lower ($P < 0.05$) hepatic cholesterol contents than those in the STW groups, and the contents decreased as low as those in the NC groups. Furthermore, the FMT + STW groups showed higher ($P < 0.05$) hepatic cholesterol contents compared to the PC + STW group. Notably, the D3-FMT + STW group had the highest hepatic cholesterol contents among all the groups (Fig. 7). In the D3-FMT + AC group, a substantial reduction ($\Delta = 60.4$ mg/g of tissue weight; $P < 0.001$) in the hepatic cholesterol was observed, which was significantly higher ($P < 0.05$) compared to the other FMT and PC groups (Fig. 7). This result suggests that because the AC treatment showed BSH activity as presented in Table 2, and the intestinal bacteria with high abundance such as *P. merdae*, *E. coprostanoligenes*, and *B. pseudolongum* have BSH activity[57]. The cholesterol in the liver might be used for synthesizing the bile acid and it might be de-conjugated by BSH of AC treatment and the microbiota, especially in the D3-FMT + AC group. Despite the high composition of the gut bacteria with BSH activity, the reason for the high hepatic cholesterol contents in the D3-FMT + STW group may be *de novo* cholesterol synthesis in the liver with the high expression HMG-CoA reductase (*hmgcr*) (Fig. 8A) and the high abundance of *R. champanellensis* in the D3-FMT + STW group. Thus, the lower cholesterol contents in the liver of D3-FMT + AC compared to the other groups might be caused by the microbiome in the gut.

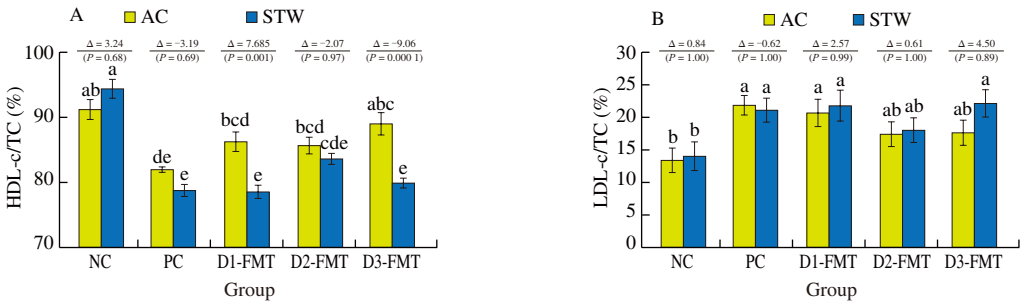


Fig. 6 Effects of FMT and AC treatment on the blood cholesterol ratios. (A) HDL-c/TC ration and (B) LDL-c/TC ratio, represented as mean ± standard deviation ($n = 10$ mice/group). Δ indicates the difference in mean values between the STW group and the AC group. [a-e] Different letters indicate significant difference ($P < 0.05$).

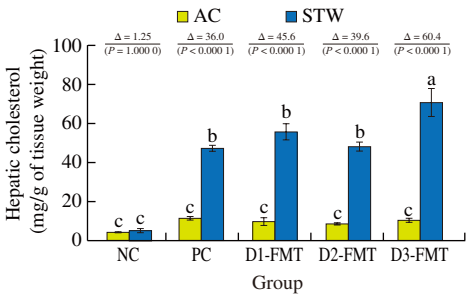


Fig. 7 Total cholesterol levels in the liver. Δ indicates the difference in mean values between the STW group and the AC group. [a-c] Different letters indicate significant difference ($P < 0.05$).

3.3.5 *Gene expression levels in liver*

Because the excretion of deconjugated bile acid in the intestine by BSH of AC treatment and certain intestinal bacteria was suggested as the cause for the lower hepatic cholesterol contents in D3-FMT + AC in a section of '*Cholesterol levels in liver*'. The mRNA expression levels associated with bile acid synthesis, cholesterol homeostasis, cholesterol synthesis and efflux, and biliary cholesterol secretion were examined (Fig. 8). *Hmgcr* is a rate-limiting enzyme that regulates cholesterol biosynthesis from HMG-CoA[67]. Thus, we measured the expression level of *hmgcr* in the liver of FMT groups and found that *hmgcr* expression was upregulated in the D3-FMT + STW group compared to that in the other experimental groups (Fig. 8A). This indicates that cholesterol synthesis in the liver increased of D3-FMT + STW group. Upregulation of LXRα, encoded by *Nr1h3*, may increase bile acid synthesis and metabolism/excretion, reverse cholesterol transport, inhibition of cholesterol biosynthesis and excretion in the liver, intestine, and peripheral tissues[68]. Pregnane X receptor (PXR, *Nr1i2*) affects genes involved in cholesterol metabolism and lipid transport in the liver[69]. A previous study reported that activation of *Nr1i2* induced hypercholesterolemia and increased plasma cholesterol concentrations in mice[70]. *Abcg5* and *Abcg8* play important roles in maintaining sterol homeostasis by transporting cholesterol from hepatocytes to bile canaliculi[71]. In addition, the rate of biliary cholesterol excretion is directly related to hepatic *Abcg5* and *Abcg8* expression[72-74]. LDL receptor (*LDLR*; *Ldlr* gene) plays a role in clearing atherogenic lipoprotein particles, such as VLDL and LDL[75]. In our study, after high cholesterol diet, *Nr1h3*, *Nr1i2*, *Abcg5*, and *Abcg8* were upregulated as shown in the STW groups except for the NC groups (Fig. 8). However, after the AC treatment, the expression levels of *Nr1h3*, *Nr1i2*, *Abcg5*, and *Abcg8* in all FMT groups decreased ($P < 0.05$) to levels similar to those of the NC group (Fig. 8). Especially, the D3-FMT + AC group showed the most significant decrease ($P < 0.05$) (Fig. 8). It indicates that a high cholesterol diet may increase serum LDL-c concentrations, and the cholesterol might be transported to the liver by the upregulation of *Ldlr*. It might cause increased hepatic cholesterol contents, as previously presented in Fig. 7. To decrease the hepatic cholesterol contents by reverse cholesterol transport or bile acid synthesis, *Nr1h3*, *Nr1i2*, *Abcg5*, and *Abcg8* should be upregulated. However, after the FMT + AC treatments, because the AC treatment decreased serum and hepatic cholesterol by its BSH activity and cholesterol assimilation, as presented in Fig. 3, *Ldlr*, *Nr1h3*, *Nr1i2*, *Abcg5*, and *Abcg8* might not need to be upregulated. Then, in the intestine, AC treatment and certain gut microflora might deconjugate bile acid, and it might be excreted by faeces. Among the FMT + AC groups, higher reductions in the expression of these genes were observed in the D3-FMT + AC group. Thus, the expression of *Cyp7a1*, which is directly related to the bile acid synthesis by converting cholesterol to bile acid in the liver, was examined, and the gene was more expressed ($P < 0.05$) in the D3-FMT group than in the other groups (Fig. 8). It indicates that the upregulated *Cyp7a1* in the D3-FMT groups might reduce additional cholesterol contents in the liver by converting cholesterol to bile acid. Considering that bile acid synthesis is the primary pathway for maintaining systemic cholesterol homeostasis, regulating of *Cyp7a1*, a rate-regulating enzyme involved in bile acid synthesis in the liver[76], is important. When *Cyp7a1* increases the synthesis of bile acids in the liver, most bile acids are conjugated to either glycine or taurine in the liver to form negatively charged bile salts, increasing their solubility[77]. The conjugated bile salts are then deconjugated by gut microorganisms with BSH in the small intestine[78]. Deconjugated bile acids are excreted by faeces, resulting in reduced serum cholesterol concentrations by increasing the demand for cholesterol to synthesise bile acid[78]. In intestine, *Eubacterium*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Bacteroides*, *Roseburia*, and *Lactobacillus* are the bacteria with BSH activity[79-81]. Especially in recent studies, *Bifidobacterium* and *Eurobacterium* showed cholesterol-lowering effect because of their BSH activity[82-83]. Bourgin et al.[84] and Thomas et al.[85] showed that the increased BSH activity in gut microbiota induced *Cyp7a1* expression to convert cholesterol

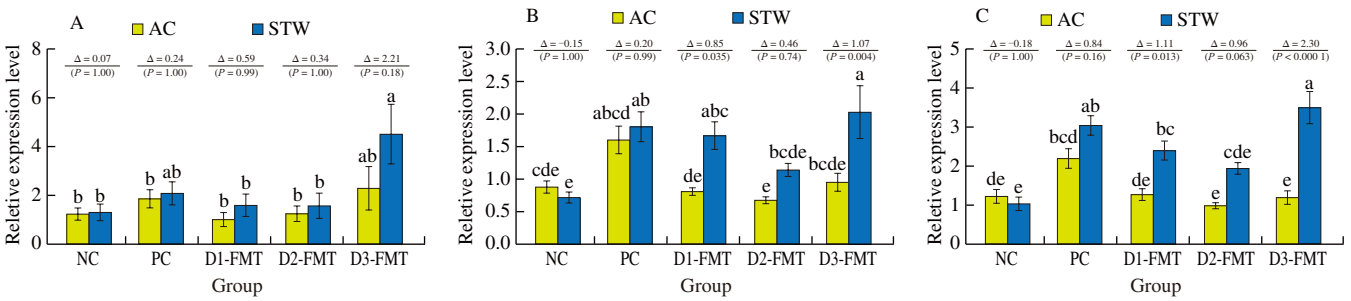


Fig. 8 Role of FMT and AC treatment on cholesterol metabolism in the liver. Gene expression levels of (A) *hmgcr*, (B) LXRα (*Nr1h3*), (C) PXR (*Nr1i2*), (D) *Abcg5*, (E) *Abcg8*, (F) *Ldlr*, and (G) *Cyp7a1* in mice following FMT and AC treatment. Δ indicates the difference in mean values between the STW group and the AC group. [a-e] Different letters indicate significant difference ($P < 0.05$).

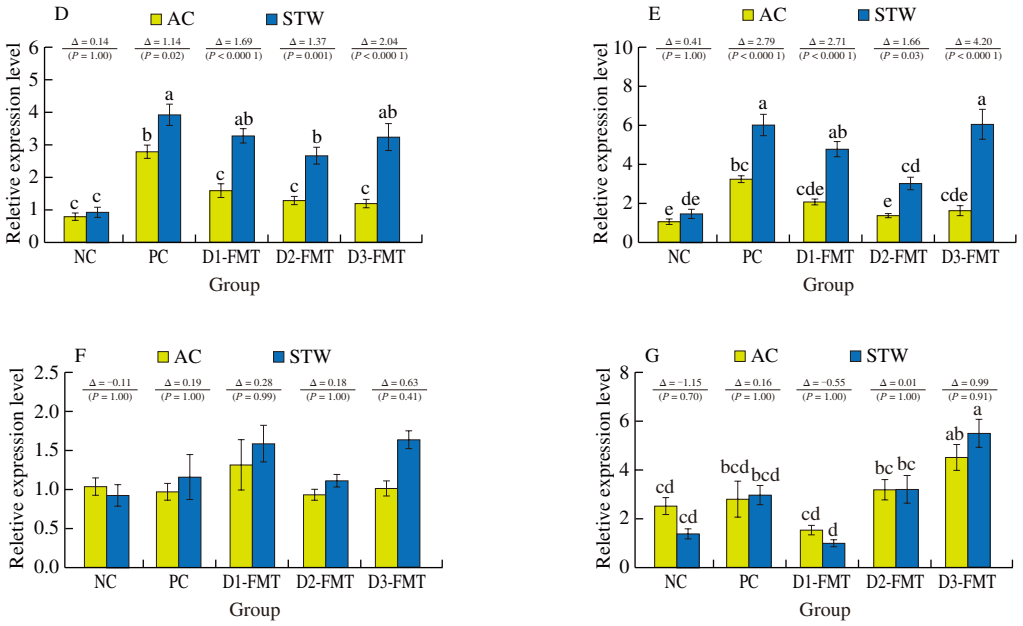


Fig. 8 (*Continued*)

to bile acid. Previous studies[82,86] showed that *Eubacterium* and *Bifidobacterium* upregulated *Cyp7a1*, which synthesized bile acid from cholesterol, and bile acid was excreted with faeces after deconjugating bile acid. This result indicates that in the D3-FMT + AC group, BSH of *Bifidobacterium* and *Eubacterium* in the intestine, as shown in Fig. 4 might upregulate *Cyp7a1*[84], which convert cholesterol to bile acid in liver, and the bile acid is deconjugated in the intestine by the BSH.

4. Conclusion

Mice colonized with different microbiomes from D1-FMT, D2-FMT and D3-FMT, and the mice groups then showed different serum LDL-c/TC ratios by the AC treatment. Especially, the D3-FMT + AC group showed the lowest serum LDL-c/TC ratio and hepatic cholesterol and higher reductions in the expression of *Ldlr*, *Nr1h3*, *Nr1i2*, *Abcg5*, and *Abcg8* genes compared to the other high cholesterol groups. Among the cholesterol metabolism-related genes, *hmgcr* and *Cyp7a1* was notably upregulated in the D3-FMT mice. *Hmgcr* is responsible for synthesizing cholesterol in the liver, and *Cyp7a1* is responsible for synthesizing bile acids from cholesterol in the liver. Up-regulations of *hmgcr* and *Cyp7a1* were particularly prominent in D3-FMT group compared to the other FMT groups. Higher proportions of *Eubacterium*, *Bifidobacterium*, and *Parabacteroides*, which had BSH activity, were observed in the intestinal microbiome of the D3-FMT + AC group. Therefore, this result suggests that the effect variation of AC treatment among individual mice might be influenced by the gut microbiome, and the intestinal bacteria with BSH activity might upregulate *Cyp7a1*, which plays a role in converting hepatic cholesterol to bile acid. The bile acid might be deconjugated by the BSH, and the deconjugated bile might be excreted by faeces. It might eventually decrease hepatic cholesterol and serum LDL-c/TC in the D3-FMT by AC treatment in the mice.

Declaration of 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.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2019R1A2C2004356).

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