

A fermented mixed tea made with camellia (*Camellia japonica*) and third-crop green tea leaves prevents nonalcoholic steatohepatitis in Sprague-Dawley rats fed a high-fat and high-cholesterol diet

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Background: Established treatments for non-alcoholic steatohepatitis (NASH) are few, thus it is imperative to develop novel dietary strategies that can prevent NASH. A fermented mixed tea (FMT) made with *Camellia japonica* (Japanese camellia) and third-crop green tea leaves by tea-rolling processing was reported to reduce body weight and adipose tissue weight in Sprague-Dawley (SD) rats. Because visceral fat is one of the most important factors for the development of hepatic steatosis, this FMT supplementation can be a candidate dietary strategy for the prevention of NASH.

Methods: Nine-week-old male SD rats were fed a high-fat and high-cholesterol (HFC) diets with or without FMT (camellia and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1) for 9 weeks (n=6–7/group). Histopathology, serology and expressions of fibrogenetic, proinflammatory, oxidative stress and lipid metabolism-related genes in the liver were evaluated.

Results: Histologically, HFC diet with FMT at a ratio of 1:5 dramatically reduced NASH progression (14%) compared to the HFC diet without FMT (100%). FMT at a ratio of 1:5 reduced hepatic steatosis due to the activation of microsomal triglyceride transfer protein, and FMT at a ratio of 1:2 reduced mRNA levels of some proinflammatory, lipid metabolism-related, fibrogenic and oxidative stress marker genes.

Conclusions: Our data suggest that FMT at a ratio of 1:5 or 1:2 likely possesses a preventive effect on NASH progression.

Keywords: Camellia leaves; green tea leaves; fermented tea; nonalcoholic steatohepatitis; high-fat and high-cholesterol (HFC) diet

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is recognized as the most common cause of chronic liver disease in many countries. In most cases NAFLD does not progress beyond nonalcoholic fatty liver (NAFL), but the liver occasionally exhibits fibrosis and necroinflammation, indicating the presence of non-alcoholic steatohepatitis (NASH) (1). Based on the “two-hit” or “multiple parallel hits” hypothesis of NASH pathogenesis (2,3), insulin resistance, oxidative stress and activation of inflammatory cytokines seem to be key features in the transition from NAFL to NASH (4). Therefore, changes in dietary habits and lifestyle have been recommended as the standard of care for NAFLD; however, these behavioral strategies have a high failure rate (4).

Green tea (*Camellia sinensis*) is a popular beverage consumed worldwide. It has been recognized for its beneficial health effect such as hypocholesterolemic, hypotriacylglycerolemic, hypoglycemic, and antimutagenic activities (5). Further, it is rich in various polyphenols such as (-)-epigallocatechin-3-gallate, (-)-epigallocatechin, (-)-epicatechin-3-gallate, and (-)-epicatechin, which are thought to be the major compounds responsible for the above biological effects (5). Evidence from *in vitro* systems and animal models suggest that green tea catechins likely play a preventive role in hepatic steatosis by decreasing intestinal lipid and carbohydrate absorption, decreasing adipose lipolysis, stimulating hepatic β -oxidation and improving insulin sensitivity. Green tea catechins also prevent the progression to NASH through their antioxidant and anti-inflammatory properties (6). Li *et al.* reported that green tea extract protected against high fat-induced liver injury and NASH by lowering hepatic lipid accumulation and nuclear factor-kappa B (NF- κ B)-dependent inflammation (7). However, Hirsch *et al.* recently reported that prolonged supplementation with green tea polyphenols exacerbated cholesterol-induced fatty liver by increasing hepatic oxidative stress and inflammation in mice (8). Teschke *et al.* also reported that green tea catechins were associated with the risk of hepatotoxicity such as drug-induced liver injury in human (9). Although green tea catechins inhibit the human hepatic and intestinal microsomal enzyme activity of various Cytochrome P450 (CYP) isoforms *in vitro*, there are no evidence that green tea catechins increase the risk of drug-induced liver injury (9,10).

Catechin oxidation in green tea leaves results in the

formation of black tea polyphenols such as theaflavins and theasinensins. These polyphenols are reported to have hypocholesterolemic and hypotriacylglycerolemic effects in cholesterol-fed rats (11). Cai *et al.* reported that a fermented dark tea (Pu-erh tea) extract ameliorated high-fat diet-induced NASH by improving lipid metabolism, inflammation and insulin resistance (12).

Camellia japonica (Japanese camellia, named “tsubaki” in Japan) is one of the best known species of the genus *Camellia*. In the wild, it is found in mainland China, Taiwan, southern Korea and southern Japan. This camellia tree is widely grown in the Goto Islands, Nagasaki Prefecture, which are Japanese islands located in the East China Sea, off the western coast of Kyushu island; however, its leaves are not effectively utilized (13). “Tsubaki” tea (*Camellia japonica*) has beneficial effects on hepatic fat metabolism as with green tea, especially its lowering effect of hepatic fatty acid synthase activity, but its taste is not so good due to the astringent leaf flavor (13). Green tea cultivation is also popular in Nagasaki Prefecture, Japan. Green tea leaves are usually harvested several times per year at tea plantations. The first crop of green tea leaves is characterized by good taste and aroma. The third crop of green tea leaves contains a relatively large amount of catechins, which imparts a bitter taste, thereby diminishing the product’s commercial value (5,14). Thus, these two leaves are not effectively utilized and are in part discarded. Recently, fermented mixed tea (FMT) made with camellia and third-crop green tea leaves at a ratio of 1:9 by tea-rolling processing, named “Goto Tsubaki-cha”, have been commercialized (13). Tamaru *et al.* reported that this mixed tea contained black tea polyphenols such as theaflavins and theasinensins in addition to catechins, and that rats fed an extract of this mixed tea had significantly lower body weight and adipose tissue weight compared to rats fed camellia tea or third-crop green tea extract alone (15). Because visceral fat is one of the most important factors for the development of hepatic steatosis, this FMT supplementation can be a candidate dietary strategy for the prevention of NAFL or NASH.

In the present study, we evaluated the preventive effect of this FMT against NASH in Sprague-Dawley (SD) rats fed a high-fat and high-cholesterol (HFC) diet. We also investigated the mechanism underlying the effects of the FMT, specifically focusing on alterations in fibrogenesis, inflammation, oxidative stress and cholesterol or lipid metabolism in the liver.

Table 1 Proximate dietary compositions

Ingredient (g/100 g)	Control	HFC	FMT1:5	FMT1:2	FMT1:1
Water	7.90	5.49	5.32	5.32	5.32
Crude protein	23.10	16.05	15.91	15.91	15.91
Crude lipid	5.10	3.54	3.51	3.51	3.51
Crude ash	5.80	4.03	3.99	3.99	3.99
Crude fiber	2.80	1.95	1.93	1.93	1.93
Nitrogen-free extract	55.30	38.43	38.10	38.10	38.10
Palm oil	0.00	28.75	28.50	28.50	28.50
Cholesterol	0.00	1.25	1.24	1.24	1.24
Sodium cholate	0.00	0.50	0.50	0.50	0.50
FMT1:5	0.00	0.00	1.00	0.00	0.00
FMT1:2	0.00	0.00	0.00	1.00	0.00
FMT1:1	0.00	0.00	0.00	0.00	1.00
Total	100.00	100.00	100.00	100.00	100.00
Energy (kcal/100 g)	359.50	508.60	504.14	504.14	504.14

Control group rats fed a MF diet which is a normal diet provided by Oriental Yeast Co. Ltd. HFC, high-fat and high-cholesterol diet; FMT1:5, FMT1:2 and FMT1:1, HFC diet supplemented with 1% (w/w) FMT made with camellia (*Camellia japonica*) and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1, respectively; FMT, fermented mixed tea.

Methods

Animals and experimental design

Eight-week-old male SD rats were purchased from Japan SLC (Hamamatsu, Japan) and housed individually in a temperature- and humidity-controlled room (22–24 °C and 50–60% relative humidity) with a 12-h light/dark cycle. After 1 week of acclimation with standard rodent chow (MF; Oriental Yeast, Tokyo, Japan) and water ad libitum, the rats were randomly divided into five groups: Control group (n=6), fed standard rodent chow (MF) as the normal diet for 9 weeks; HFC group (n=6), fed an HFC diet for 9 weeks; FMT1:5 group (n=7), fed a HFC diet supplemented with 1% (w/w) FMT (FMT) made with camellia (*Camellia japonica*) and third-crop green tea leaves at a ratio of 1:5 for 9 weeks; FMT1:2 group (n=6), fed a HFC diet supplemented with 1% (w/w) FMT at a ratio of 1:2 for 9 weeks; and FMT1:1 group (n=7), fed a HFC diet supplemented with 1% FMT at a ratio of 1:1 for 9 weeks. The HFC diet was prepared by mixing the MF with 30% (w/w) palm oil, 1.25% (w/w) cholesterol, and 0.5% (w/w) sodium cholate (16,17). FMT were provided by the Nagasaki Prefectural Agricultural and Forestry Experiment Station, Higashisonogi Tea

Branch, Nagasaki, Japan, as described previously (15). The proximate dietary compositions of each diet fed to rats are shown in *Table 1*. Daily energy intake and body weight were monitored throughout the study.

At 18 weeks of age, the rats were fasted for 8 h and sacrificed under anesthesia with pentobarbital sodium. Organs were harvested and blood was collected from the inferior vena cava. Liver tissues were either placed in 10% neutral buffered formalin or snap frozen in liquid nitrogen and stored at –80 °C.

Histopathological assessment of the liver

Liver tissues stored in 10% neutral-buffered formalin were embedded in paraffin, sectioned at 4 µm, and processed for hematoxylin-eosin (HE) staining for histopathological examination. Histological steatosis [0–3], lobular inflammation [0–3] and hepatocyte ballooning [0–2] were assessed semiquantitatively to determine the NAFLD activity score (NAS) according to the criteria proposed by Kleiner *et al.* (18). The final NAS values ranged from 0 to 8. NAS scores ≥5 and ≤2 were considered diagnostic and not diagnostic, respectively, for steatohepatitis. Liver fibrosis

Table 2 Primer sequences for real-time PCR

Primer	Sequence (5' to 3')
COL1A1	Forward: GCGTAGCCTACATGGACCAA
	Reverse: AAGTTCGGGTGTGACTCGTG
TGF- β	Forward: CTTTGTACAACAGCACCCGC
	Reverse: TAGATTGCGTTGTTGCGGTC
α -SMA	Forward: GCCAAGAAGACATCCCTGAAGT
	Reverse: TGTGGCAGATACAGATCAAGCAT
TNF- α	Forward: TGATCGGTCCCAACAAGGA
	Reverse: TGGGCTACGGGCTTGTC
MCP-1	Forward: TCTGTCACGCTTCTGGGCTGT
	Reverse: GGGGCATTAAGTGCATCTGGCTGAG
IL-1 β	Forward: CTCCAGTCAGGCTTCCTTGTG
	Reverse: GGTCACTCTCCTCACTGTGAAA
IL-6	Forward: GATACCACCCACAACAGACCAGTA
	Reverse: TGCACAACCTTTTTCTCATTCCA
NF- κ B	Forward: TGACATCATCAACATGAGAAACGA
	Reverse: CCCCAACCTCAGCAAGTC
CYP2E1	Forward: CCCATCCTTGGGAACATTTTT
	Reverse: GCCAAGGTGCAGTGTGAACA
HO-1	Forward: CACAGGGTGACAGAAGAGGCTAA
	Reverse: GGGACTCTGGTCTTTGTGTTCCCT
GPX-1	Forward: GCTGCTCATTGAGAATGTCCG
	Reverse: GAATCTCTCATTCTTGCCATT
MnSOD	Forward: GACCTGCCTTACGACTATG
	Reverse: TACTTCTCCTCGGTGACG
LXR- α	Forward: CAGGACCAGCTCCAAGTAGA
	Reverse: GAACATCAGTCGGTCTGTGG
MTP	Forward: CAAGCTCAAGGCAGTGGTTG
	Reverse: AGCAGGTACATCGTGGTGTG
FAS	Forward: CAACATTGACGCCAGTTCCG
	Reverse: TTCGAGCCAGTGTCTCCAC
GPAT	Forward: GCTACCTGAAGGTGAGCCAG
	Reverse: AGGTACTCAGACTCCGGGAC
CPT-1	Forward: AACCTCGGACCCAAATTGC
	Reverse: GGCCCCGCGAGGTAGATATAT
AOX	Forward: CCACTGAACAAAACAGAGGTCC
	Reverse: GTCCCAGGGAACTTCAAAGC

[0–4] was also assessed by Azan staining. Each specimen was also assigned to one of four histopathological subgroups: type 1, fatty liver alone; type 2, fat accumulation and lobular inflammation; type 3, fat accumulation and ballooning degeneration; type 4, fat accumulation, ballooning degeneration, and either Mallory hyaline or fibrosis, according to the criteria proposed by Matteoni *et al.* (19). All histopathological examinations were performed by a pathologist (K.T.) who was blinded to the experimental and serological data.

Serum biochemical analysis

Serum triglyceride (TG), total cholesterol (TC), glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined by Triglyceride E test Wako, Cholesterol E test Wako, Glucose C II test Wako, and Transaminase C II test Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Serum insulin, leptin, and adiponectin levels were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan), a mouse/rat leptin ELISA kit (Morinaga Institute of Biological Science Inc.), and a mouse/rat adiponectin ELISA kit (Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan), respectively.

Quantification of mRNA using real-time PCR

Total RNA from the liver was extracted using RNAiso Plus (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA templates using a commercial kit (PrimeScript RT Master Mix, Takara Bio). Real-time PCR analysis was performed as described previously (16,17). Specific primers were designed using the primer designing tool Primer-BLAST [National Center for Biotechnology Information (NCBI), Bethesda, MD, USA] and were synthesized by Greiner Bio-One Japan (Tokyo, Japan; Table 2). mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a house-keeping gene. For studies in rats, hepatic expressions of genes involved in fibrosis [collagen type I alpha 1 (COL1A1), transforming growth factor- β (TGF- β), and α -smooth muscle actin (α -SMA)], inflammation [tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and nuclear factor- κ B (NF- κ B)], oxidative stress [cytochrome P450 family 2 subfamily E polypeptide

Table 3 Cumulative energy intake, body weight and relative organ weights at 18 weeks of age

Item/group	Control	HFC	FMT1:5	FMT1:2	FMT1:1
Cumulative energy intake (kcal)	5,039±114 ^a	6,338±241 ^b	6,757±168 ^b	6,677±136 ^b	6,471±211 ^b
Final body weight (g)	495±14	543±23	564±18	563±16	545±15
Body weight gain (g)	197±8	245±20	262±17	265±13	243±15
Food efficacy (g/kcal)	0.039±0.001	0.038±0.002	0.039±0.002	0.040±0.001	0.037±0.004
Liver weight/body weight (%)	2.8±0.1 ^a	6.1±0.1 ^b	6.0±0.2 ^b	5.7±0.1 ^{bc}	5.3±0.2 ^c
Epididymal fat pad weight/body weight (%)	1.7±0.1	2.0±0.2	2.2±0.2	2.2±0.2	2.2±0.1

Values are expressed as means ± SE, n=6–7/group. ^{abc}, Values not sharing the same lowercase letter in a row are significantly different among groups (P<0.05). Food efficacy was calculated by body weight gain (g)/cumulative energy intake (kcal). HFC, high-fat and high-cholesterol diet; FMT1:5, FMT1:2 and FMT1:1, HFC diet supplemented with 1% (w/w) FMT made with camellia (*Camellia japonica*) and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1, respectively; FMT, fermented mixed tea.

1 (CYP2E1), heme oxygenase-1 (HO-1), glutathione peroxidase-1 (GPX-1), and manganese superoxide dismutase (MnSOD)], cholesterol metabolism [liver X receptor- α (LXR- α)], and lipid metabolism [microsomal triglyceride transfer protein (MTP), fatty acid synthase (FAS), glycerol-3-phosphate acyltransferase (GPAT), carnitine palmitoyltransferase-1 (CPT-1), and acyl CoA oxidase (AOX)] were quantified. All data were expressed as fold changes compared to Control group expression.

Statistical analysis

All values were expressed as mean ± standard error (SE). Differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA), followed by Scheffe's post hoc test, chi-square test, or Fisher's exact probability test. All analyses were performed using IBM SPSS statistics software program, version 23 (IBM Co., Somers, NY, USA) on a Windows computer. A P value of less than 0.05 was considered to be statistically significant.

Results

Cumulative energy intake, body weight and relative organ weight

Cumulative energy intake was significantly higher in the HFC, FMT1:5, FMT1:2 and FMT1:1 groups than the Control group (P<0.01), whereas body weight at 18 weeks of age, weight gain during the 9-week study and food efficacy were not significantly different among the groups. The liver weight/body weight ratio at 18 weeks of age was

significantly higher in the HFC, FMT1:5, FMT1:2 and FMT1:1 groups than the Control group (P<0.01), and this ratio was significantly lower in the FMT1:1 group than the HFC and FMT1:5 groups (P<0.01). The epididymal fat pad weight/body weight ratio was not significantly different among the groups (Table 3).

Histopathological findings of the liver

Representative histopathology of the rat liver and histological assessments is shown in Figure 1, Table 4 and Figure S1. No obvious findings of hepatic steatosis, lobular inflammation, hepatocyte ballooning or fibrosis were seen in any of the rats in the Control group. In contrast, severe steatosis (score 3) and moderate lobular inflammation were observed in all six rats of the HFC group. Hepatocyte ballooning and portal/periportal or bridging fibrosis (score 2 or 3) was observed in three and one of the six rats in the HFC group, respectively. In the FMT1:5 group, moderate steatosis (score 2), mild lobular inflammation (score 1) and no hepatocyte ballooning (score 0) was observed in 5 (71%), 3 (43%) and 6 (86%) of seven rats, respectively. In the FMT1:2 group, moderate steatosis (score 2), mild lobular inflammation (score 1) and no hepatocyte ballooning (score 0) was observed in 2 (33%), 1 (17%) and 5 (83%) of six rats, respectively. No portal/periportal or bridging fibrosis (score 2 or 3) was seen in any of the rats in the FMT1:5 and FMT1:2 groups. In the FMT1:1 group, the results of hepatic steatosis, lobular inflammation, hepatocyte ballooning or fibrosis were similar to those in the HFC group. According to the NAS (18), none of six rats (0%), all of six rats (100%), one of seven rats (14%), four of six rats (67%) and six of seven rats (86%) in

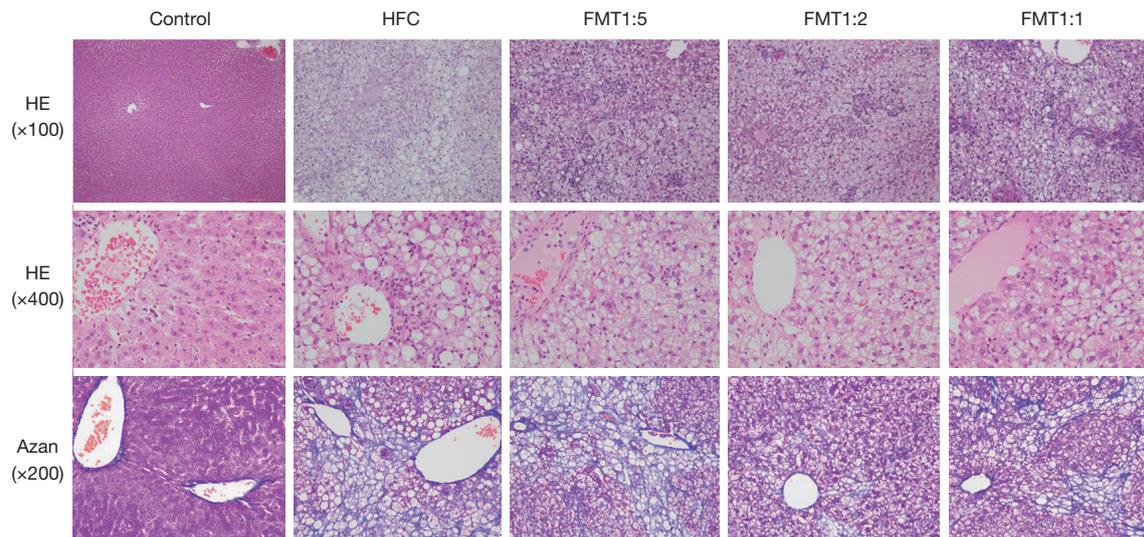


Figure 1 Representative histopathology of the rat liver in the Control, HFC, FMT1:5, FMT1:2 and FMT1:1 groups at 18 weeks of age (upper panels: HE-stained section; original magnification, $\times 100$, middle panels: HE-stained section; original magnification, $\times 400$, lower panels: Azan-stained section; original magnification, $\times 200$). HFC, high-fat and high-cholesterol diet; FMT1:5, FMT1:2 and FMT1:5, the HFC diet supplemented with 1% (w/w) FMT made with camellia (*Camellia japonica*) and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1, respectively; FMT, fermented mixed tea.

Table 4 Histopathological assessment of the livers according to the NASH Clinical Research Network Scoring System proposed by Kleiner *et al.* (18) and subgroups proposed by Matteoni *et al.* (19) at 18 weeks of age

Item/group	Score	Control	HFC	FMT1:5	FMT1:2	FMT1:1
Steatosis	0	6	0	0	0	0
	2	0	0	5	2	0
	3	0	6	2	4	7
Lobular inflammation	0	6	0	0	0	0
	1	0	0	3	1	1
	2	0	6	4	4	5
	3	0	0	0	1	1
Hepatocyte ballooning	0	6	3	6	5	4
	1	0	3	1	1	3
NAFLD activity score (NAS)*	0–4	6	0	6	2	1
	5–8	0	6	1	4	6
Fibrosis	0–1	6	5	7	6	6
	2–3	0	1	0	0	1
Subgroup type**	Type 0	6	0	0	0	0
	Type 2	0	3	6	5	4
	Type 4	0	3	1	1	3

Values are expressed as the number of rats. *, NAFLD activity score (NAS) was defined as the unweighted sum of scores for steatosis, lobular inflammation and hepatocyte ballooning for a final score ranging from 0 to 8. NAS scores ≥ 5 were considered diagnostic for steatohepatitis (18). **, Subgroups were according to the criteria proposed by Matteoni *et al.* (19). HFC, high-fat and high-cholesterol diet; FMT1:5, FMT1:2 and FMT1:5, HFC diet supplemented with 1% (w/w) FMT made with camellia (*Camellia japonica*) and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1, respectively; FMT, fermented mixed tea.

Table 5 Serum biochemical parameters at 18 weeks of age

Parameter/group	Control	HFC	FMT1:5	FMT1:2	FMT1:1
Triglyceride (mg/dL)	52±7	73±23	55±6	35±3	48±5
Total cholesterol (mg/dL)	63±3 ^{ab}	83±3 ^c	79±7 ^{ac}	58±4 ^b	69±3 ^{abc}
Glucose (mg/dL)	145±6	165±18	171±6	185±10	173±16
Insulin (ng/mL)	3.6±0.8	4.5±0.6	2.8±0.5	3.6±0.6	4.2±0.6
Leptin (ng/mL)	3.7±0.4	7.3±1.0	5.2±0.7	6.1±0.5	7.3±1.3
AST (IU/L)	100±17	159±35	165±29	140±26	157±9
ALT (IU/L)	14±2 ^a	41±10 ^{ab}	43±7 ^{ab}	40±10 ^{ab}	46±3 ^b

Values are expressed as means ± SE, n=6–7/group. ^{abc}, Values not sharing the same lowercase letter in a row are significantly different among groups (P<0.05). ALT, alanine aminotransferase; AST, aspartate aminotransferase; HFC, high-fat and high-cholesterol diet; FMT1:5, FMT1:2 and FMT1:1, the HFC diet supplemented with 1% (w/w) FMT made with camellia (*Camellia japonica*) and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1, respectively; FMT, fermented mixed tea.

the Control, HFC, FMT1:5, FMT1:2 and FMT1:1 groups, respectively, were diagnosed with NASH (i.e., having a NAS of 5 or greater). According to the criteria proposed by Matteoni *et al.* (19), three of six (50%) HFC group rats and three of seven (43%) FMT1:1 group rats were type 4, representing NASH, whereas one of seven (14%) FMT1:5 group rats and one of six (17%) FMT1:2 group rats had definitive evidence of NASH.

Serum biochemical parameters

Serum TG levels were not significantly different among the groups, whereas serum TC levels were significantly higher in the HFC group than the Control group (P=0.049) and were significantly lower in the FMT1:2 group than the HFC and FMT1:5 groups (P=0.016 and 0.049, respectively). Serum glucose levels did not significantly differ among the groups. Serum insulin and leptin levels both tended to be lower in the FMT groups in a green tea extract dose-dependent manner compared to the HFC group, although the levels did not significantly differ among the groups. There were no significant difference in serum AST levels among the groups, but serum ALT levels were significantly higher in the FMT1:1 group than the Control group (P=0.038) (Table 5).

Hepatic mRNA expression

To elucidate the molecular mechanisms of the effect of FMT on NASH, we evaluated key molecular markers of fibrosis, inflammation, oxidative stress, and cholesterol or lipid metabolism in the liver. The mRNA levels of *COL1A1*

and α -SMA, which are both responsible for fibrogenesis, tended to be lower in the FMT1:2 group and higher in the FMT1:5 group than the HFC group, although differences were not statistically significant. The mRNA levels of *IL-1 β* , *IL-6* and *NF- κ B*, which are involved in inflammation, also tended to be lower in the FMT1:2 group and higher in the FMT1:5 group than the HFC group, although differences were not statistically significant. Also, the mRNA level of *HO-1*, which is a marker of oxidative stress, tended to be lower in the FMT1:2 group than the HFC group, although difference was not statistically significant. The mRNA level of *MnSOD*, which is a member of the iron/manganese superoxide dismutase family, tended to be lower in the FMT1:2 and FMT1:5 groups than the HFC group, although differences were not statistically significant. The mRNA level of MTP, which is a rate-limiting protein in the synthesis and excretion of very-low-density lipoprotein from the liver, tended to be higher in the FMT1:5 than the HFC group, although difference was not statistically significant (Figure 2).

Discussion

Established treatments for NAFLD/NASH are few, thus it is vital to develop and validate novel dietary strategies to prevent or attenuate the development of hepatic steatosis and its progression to NASH (6). In the present study, HFC diet supplemented with FMT at a ratio of 1:5 dramatically reduced NASH progression (14%) diagnosed according to the NAS (18) compared to HFC diet without FMT (100%). Serum glucose levels did not significantly differ among the

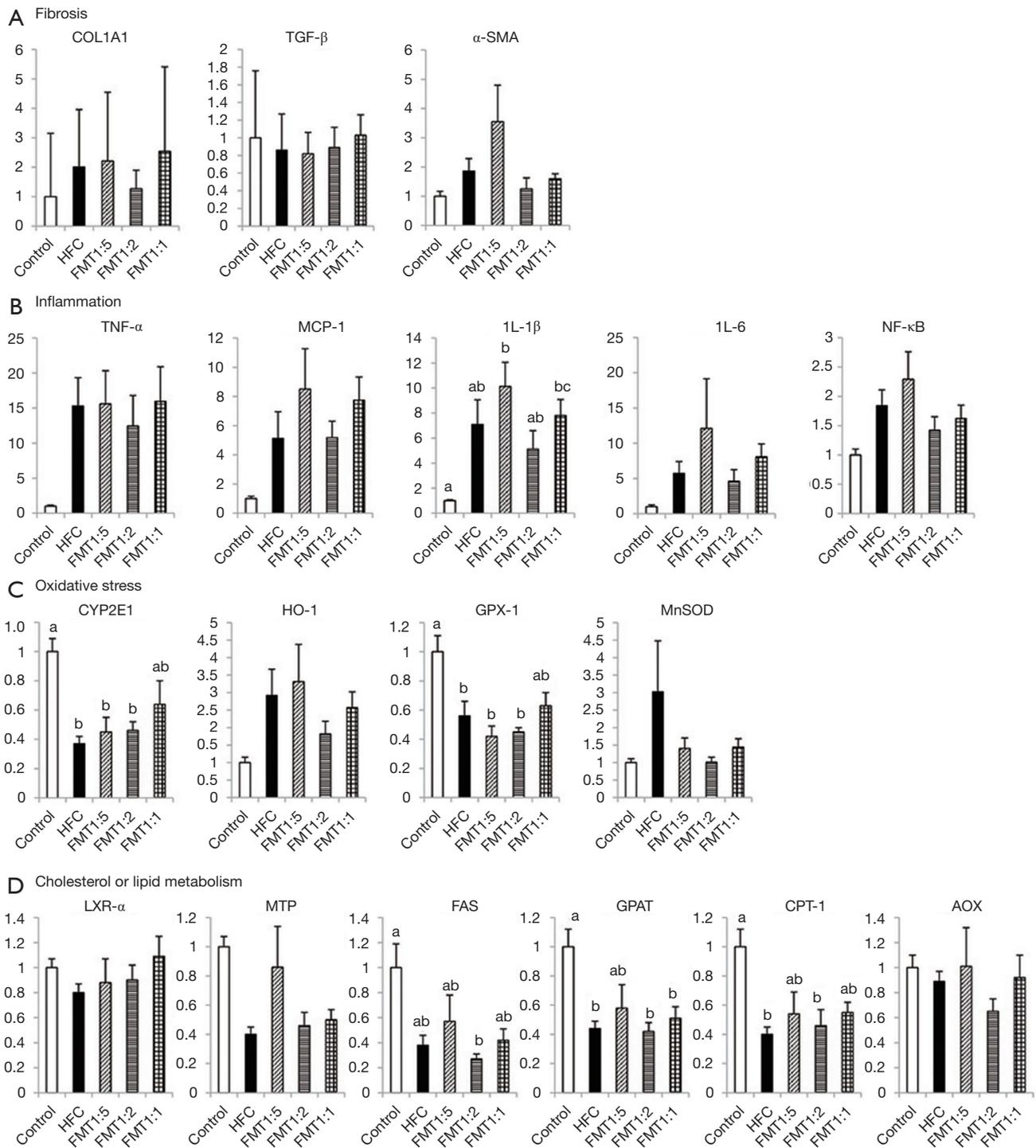


Figure 2 Hepatic gene expression involved in (A) fibrosis, (B) inflammation, (C) oxidative stress, and (D) cholesterol or lipid metabolism in the Control, HFC, FMT1:5, FMT1:2 and FMT1:1 groups. mRNA levels were expressed relative to the Control group. Values are expressed as means \pm SE; $n=6-7$ /group. abc, Values not sharing the same lowercase letter are significantly different among groups ($P<0.05$). HFC, high-fat and high-cholesterol diet; FMT1:5, FMT1:2 and FMT1:5, the HFC diet supplemented with 1% (w/w) FMT made with camellia (*Camellia japonica*) and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1, respectively; FMT, fermented mixed tea.

groups, but serum insulin levels tended to be lower in the FMT groups in a green tea extract dose-dependent manner compared to the HFC group, suggesting that green tea leaves reduce insulin resistance. Serum leptin levels also tended to be lower in the FMT groups in a green tea extract dose-dependent manner compared to the HFC group. Insulin resistance is an early event leading to NASH (7), and a high level of serum leptin may contribute to hepatic inflammation and fibrosis in NAFLD (20). Therefore, FMT at a ratio of 1:5 may have a preventive effect on NASH progression.

In the FMT1:5 group, histopathological scores of steatosis were lower compared to the HFC group in the present study. This result was partly due to the synthesis and excretion of very-low-density lipoprotein from the liver, as the mRNA levels of MTP tended to be higher in the FMT1:5 group than the HFC group, although difference was not statistically significant. Also, the mRNA levels of *MnSOD*, an oxidative stress marker, tended to be lower in the FMT1:5 group than the HFC group, although difference was not statistically significant. However, mRNA levels of proinflammatory genes including *MCP-1*, *IL-1 β* , *IL-6* and *NF- κ B*, and fibrogenic gene *α -SMA* tended to be higher in the FMT1:5 group than the HFC group. These results were inconsistent with the lower histopathological scores of lobular inflammation.

On the other hand, HFC diet supplemented with FMT at ratios of 1:2 and 1:1 did not reveal obvious effects on NASH prevention (67% and 86%, respectively) diagnosed according to the NAS (16) compared to the HFC fed rats (100%) in the present study. However, the mRNA levels of proinflammatory genes including *TNF- α* , *IL-1 β* , *IL-6* and *NF- κ B*, and the lipid metabolism-related genes *FAS* and *AOX* tended to be lower in the FMT1:2 group than the HFC group. Moreover, mRNA levels of the fibrogenic genes *COL1A1* and *α -SMA*, and the oxidative stress marker genes *HO-1* and *MnSOD* also tended to be lower in the FMT1:2 group than the HFC group. These results may suggest that FMT at a ratio of 1:2 may also have a potential preventive effect on NASH progression.

There were several limitations in the present study. First, the study lacked a group fed HFC diet supplemented exclusively with green tea extract. Because the NAS, serum insulin and leptin levels tended to be lower in the FMT groups in a green tea extract dose-dependent manner compared to the HFC group, green tea leaves may possess a preventive effect on NASH progression. However, our preliminary study showed that HFC diet supplemented with FMT at a ratio of 1:9 had no effect on NASH prevention

(unpublished data). Therefore, FMT at a ratio around 1:5 or 1:2 may be a better compounding ratio for the production of black tea polyphenols such as theaflavins and theasinensins in addition to catechins, as reported by Tamaru *et al.* (15). Second, most of the differences observed in the present study were not statistically significant. Especially, FMT appeared to have no effect on serum AST and ALT concentrations, which are the commonly used marker for hepatocellular injury including NASH (21). The reason is uncertain, but may be due to the no effect on the histopathological lobular inflammation score between HFC and FMT groups, or hepatotoxic effect of FMT. Third, our data suggest that human equivalent dose would be 20–25 g of FMT, which is a large quantity of tea consumption (more than 10 cups of tea per day). The optimal compound (dose) amount of FMT or dosing period should be investigated in a future study.

Conclusions

In the present study, FMT at a ratio of 1:5 or 1:2 likely possessed a preventive effect on NASH progression. FMT at a ratio of 1:5 reduced hepatic steatosis due to the activation of MTP, and FMT at a ratio of 1:2 reduced mRNA levels of some proinflammatory, lipid metabolism-related, fibrogenic and oxidative stress marker genes, suggested that FMT had a preventive effect on NASH progression. In light of the conflicting results between the histopathological and gene expression analyses, further studies are needed to fully elucidate the responsible mechanism(s).

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Footnote

Conflict of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: All procedures performed on the animals were approved by the Animal Use Committee of the University of Nagasaki (No. 27-14), and the animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals, University of Nagasaki.

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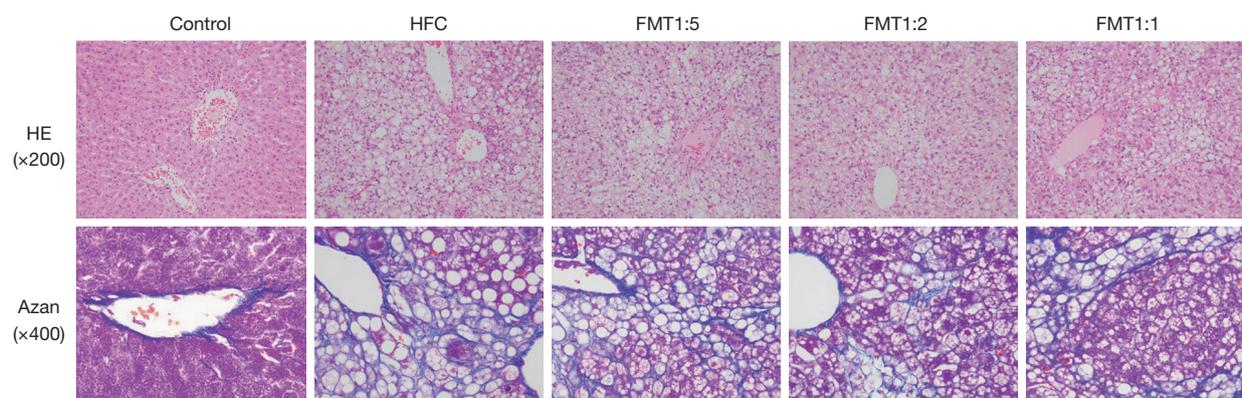


Figure S1 Representative histopathology of the rat liver in the Control, HFC, FMT1:5, FMT1:2 and FMT1:1 groups at 18 weeks of age (upper panels: HE-stained section; original magnification, $\times 200$, lower panels: Azan-stained section; original magnification, $\times 400$). HFC, high-fat and high-cholesterol diet; FMT1:5, FMT1:2 and FMT1:1, the HFC diet supplemented with 1% (w/w) FMT made with camellia (*Camellia japonica*) and third-crop green tea leaves at ratios of 1:5, 1:2 and 1:1, respectively; FMT, fermented mixed tea.