British Journal of Nutrition (2022), 127, 1621–1630 doi:10.1017/S0007114521002610

Dietary raffinose ameliorates hepatic lipid accumulation induced by cholic acid via modulation of enterohepatic bile acid circulation in rats

Kenta Maegawa, Haruka Koyama, Satoru Fukiya, Atsushi Yokota, Koichiro Ueda and Satoshi Ishizuka*

Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

(Submitted 30 November 2020 – Final revision received 21 June 2021 – Accepted 8 July 2021 – First published online 14 July 2021)

Abstract

Enterohepatic circulation of 12*α*-hydroxylated (12*α*OH) bile acid (BA) is enhanced depending on the energy intake in high-fat diet-fed rats. Such BA metabolism can be reproduced using a diet supplemented with cholic acid (CA), which also induces simple steatosis, without inflammation and fibrosis, accompanied by some other symptoms that are frequently observed in the condition of non-alcoholic fatty liver in rats. We investigated whether supplementation of the diet with raffinose (Raf) improves hepatic lipid accumulation induced by the CA-fed condition in rats. After acclimation to the AIN-93-based control diet, male Wistar rats were fed diets supplemented with a combination of Raf (30 g/kg diet) and/or CA (0·5 g/kg diet) for 4 weeks. Dietary Raf normalised hepatic TAG levels (two-way ANOVA $P < 0{\cdot}001$ for CA, $P = 0{\cdot}02$ for Raf and $P = 0{\cdot}004$ for interaction) in the CA-supplemented diet-fed rats. Dietary Raf supplementation reduced hepatic 12*α*OH BA concentration (two-way ANOVA $P < 0{\cdot}001$ for CA, $P = 0{\cdot}003$ for Raf and $P = 0{\cdot}03$ for interaction). The concentration of 12*α*OH BA was reduced in the aortic and portal plasma. Raf supplementation increased acetic acid concentration in the caecal contents (two-way ANOVA $P = 0{\cdot}001$ as a main effect). Multiple regression analysis revealed that concentrations of aortic 12*α*OH BA and caecal acetic acid could serve as predictors of hepatic TAG concentration ($R^2 = 0{\cdot}55$, $P < 0{\cdot}001$). However, Raf did not decrease the secondary 12*α*OH BA concentration in the caecal contents as well as the transaminase activity in the CA diet-fed rats. These results imply that dietary Raf normalises hepatic lipid accumulation via suppression of enterohepatic 12*α*OH BA circulation.

Key words: **Raffinose: Bile acid: Hepatic lipids: Enterohepatic circulation**

Excessive diet consumption induces obesity, type 2 diabetes mellitus and the metabolic syndrome, risk factors associated with non-alcoholic fatty liver disease (NAFLD), which includes fatty liver diseases in individuals without significant alcohol consumption, ranging from fatty liver to steatohepatitis and cirrhosis[1]. The global prevalence of NAFLD in patients with type 2 diabetes mellitus is 55·5 %[2], while 29 and 25 % of the entire population in Japan[3] and the world[4], respectively, are currently believed to have NAFLD. NAFLD can progress to cirrhosis and is the most rapidly growing cause of hepatocellular carcinoma[5]. Thus, in order to prevent the other liver diseases, there is an urgent need to find strategies that reduce the risk of NAFLD.

In rat experiments, excessive energy consumption in the form of a high-fat diet enhances the secretion of 12*α*-hydroxylated (12*α*OH) bile acid (BA)[6]. In addition, 12*α*OH BA concentrations in the liver, portal blood and faeces are associated with hepatic lipid accumulation[7]. Such an association has also been reported between 12*α*OH BA and fatty liver index in a clinical study[8]. BA synthesised from cholesterol (Chol) are secreted into the duodenum. Cholic acid (CA; 12*α*OH BA) enhances the absorption of dietary Chol, but chenodeoxycholic acid (non-12*α*OH BA) does not[9], which suggests that 12*α*OH BA contribute to the absorption of hydrophobic nutrients. In a previous study carried out by our group on rats[10], dietary supplementation of the diet with 0·5 g/kg CA increased the proportion of 12*α*OH BA in the portal plasma, liver, caecal contents and faeces. Also, the dietary CA supplementation in rats led to various abnormalities, such as the development of hepatic steatosis without obesity, hepatic injury and dysbiosis in the gut microbiota. These observations suggest that an increase in the levels of 12*α*OH BA in the body associates hepatic steatosis with related diseases.

In addition to dietary energetic restriction and exercise, oligosaccharides also may serve as an effective way to prevent hepatic steatosis. Raffinose (Raf), an indigestible oligosaccharide, is a

Abbreviations: Abcc2, ATP-binding cassette subfamily C member 2; Asbt, apical Na-dependent bile acid transporter; BA, bile acid; CA, cholic acid; Chol, cholesterol; Cidea, cell death-inducing DNA fragmentation factor-α-like effector A; *Cpt1*, carnitine O-palmitoyltransferase 1; DCA, deoxycholic acid; Fasn, fatty acid synthase; 12αOH, 12α-hydroxylated; NAFLD, non-alcoholic fatty liver disease; *Ost*, organic solute transporter subunit; Raf, raffinose; Srebp1, sterol regulatory element binding protein-1.

* **Corresponding author:** Satoshi Ishizuka, email zuka@chem.agr.hokudai.ac.jp

British Journal of Nutrition

trisaccharide composed of galactose, glucose and fructose (*β*-D-fructofuranosyl-*O*-*α*-D-galactopyranosyl-(1,6)-*α*-D-glucopyranoside). Dietary Raf increases the proportion of *Bifidobacterium*, while reducing that of *Clostridium* in the microbiota of rats[11], which may contribute to reducing the conversion of BA to toxic secondary BA[12]. An increase in *Bifidobacterium* upon oral Raf administration has also been confirmed in humans[11]. Some indigestible oligosaccharides increase BA excretion[13,14], and a diet that includes an unpurified Raf-containing supplement lowers hepatic BA concentration as well as hepatic TAG concentration in rats fed a high-fat diet[15]. However, the mechanism in which dietary Raf reduces hepatic TAG concentration remains unknown. In our previous study, we observed association between hepatic TAG concentration and 12*α*OH BA levels in enterohepatic circulation[7]. Those observations suggest that dietary Raf reduces hepatic TAG via modulation of BA metabolism in enterohepatic circulation.

We investigated whether Raf ingestion alleviates hepatic lipid accumulation by modulating BA metabolism in a novel steatosis model with dietary CA supplementation.

Methods

Animal and diets

The animal experiments were approved by the Institutional Animal Care and Use Committee of the National Corporation Hokkaido University (approval number: 17-0119), and all the animals were maintained according to the Hokkaido University Manual for Implementing Animal Experimentation. Male Wistar rats (*n* 24; 3 weeks old; Japan SLC Inc.) were individually housed in a controlled environment at 22 (SEM 2)°C and 55 (SEM 5) % humidity. The light period was from 08.00 to 20.00 hours.

The rats had free access to food and water for the entire study period. After acclimation with a control diet based on AIN-93G[16] for 2 weeks, the rats were divided into four dietary groups (*n* 6 each): control, CA supplementation (CA; 0·5 g CA/kg diet), Raf supplementation (Raf; 30 g Raf/kg diet) and a combined supplementation with CA and Raf (CA + Raf; 0·5 g CA/kg diet and 30 g Raf/kg diet) to have comparable average body weights based on measurements on the day when the test diet was initiated, as shown in Table 1. In this study, the investigators and outcome assessors were not blinded to the intervention. We confirmed that deoxycholic acid (DCA) is the major form of 12*α*OH BA in human faeces[17]. The range of DCA concentration is from 0·07 to 0·73 mmol/l in human faecal water on a high-fat diet[18], and it is possible to damage bacterial membrane in the DCA levels[19]. In our previous observation[20], DCA concentration was calculated from 0·07 to 0·98 μmol/g caecal contents in rats fed diet supplemented with CA at 0·5 g/kg diet. In the present study, we used this CA supplementation level to mimic BA environment in those observations. A standard supplementation level of dietary fibre is 50 g/kg diet according to AIN-93 formulation[16]. We observed increase in the proportion of bifidobacteria accompanied by reduction of pH in caecal contents and an increase in caecal tissue weight in rats fed a diet supplemented with Raf at 30 g/kg and crystalline cellulose at 50 g/kg diet (80 g fibre source/kg diet in total)[11,21]. The supplementation levels of indigestible saccharides correspond daily dietary fibre intake in humans (0·5–8·0 g/MJ diet)[22].

Table 1. Diet compositions

	Control	CA	Raf	CA + Raf
	g/kg diet			
Casein*	200	200	200	200
Dextrin†	529·5	529·5	499·5	499·5
Sucrose‡	100	99·5	100	99·5
Soyabean oil§	70	70	70	70
Cellulose‖	50	50	50	50
Mineral mixture¶	35	35	35	35
Vitamin mixture**	10	10	10	10
L-Cystine††	3	3	3	3
Choline chloride††	2·5	2·5	2·5	2·5
Sodium cholate††	–	0·5	–	0·5
Raffinose‡	–	–	30	30

* NZMP Acid Casein (Fonterra Co-Operative Group Limited).
† TK-16 (Matsutani Chemical Industry Co. Ltd).
‡ Nippon Beet Sugar Manufacturing Co. Ltd.
§ J-Oil Mills, Inc.
‖ Crystalline cellulose (Ceolus PH-102, Asahi Kasei Chemicals Corp.).
¶ AIN-93G Mineral mixture.
** AIN-93 Vitamin mixture.
†† Wako Pure Chemical Industries, Ltd.

Body weights and food intake were measured every 2 d, and the entire experimental period was 4 weeks long. Faecal samples were collected from days 27 to 28 and stored at −30°C for analysis of BA composition and energy content. At the end of the experimental period, portal and aortic blood were collected under anaesthesia via an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight, Somnopentyl, Kyoritsuseiyaku Corporation) and added to heparin (final concentration at 50 U/ml) and aprotinin (final concentration at 500 KIU/ml). After the rats were euthanised by exsanguination, the caecum was collected and the weights of the whole caecum and the caecal contents were measured. The caecal contents were diluted four times with deionised water and homogenised with a Teflon homogeniser. The pH levels of the homogenates were measured using a semiconducting electrode (Argus ISFET pH sensor; Sentron). Supernatants of the caecal contents were kept in liquid N_2 and stored at −80°C for organic acid analysis. Ileal contents and mucosa were collected and stored at −30°C and −80°C for BA analysis and real-time quantitative PCR, respectively. Liver and epididymal adipose tissues were collected and weighed. Blood plasma was separated using centrifugation at 2000 ***g*** for 10 min at 4°C. The plasma and liver samples were kept in liquid N_2 and stored at −80°C until analysis.

Biochemical analyses

Lipid extraction from liver and faecal samples was performed as previously described[23]. The activities of plasma aspartate aminotransferase and alanine aminotransferase were measured using a transaminase CII-test Wako kit (Wako Pure Chemical Industries). Chol and TAG levels were measured using a Cholesterol E-test kit and Triglyceride E-test kit (both from Wako), respectively. Hepatic NEFA concentration was measured using a NEFA C-test kit (Wako).

Bile acid analysis

BA extraction and HPLC separation were performed as previously described[24–26]. Individual BA concentration was measured using nordeoxycholic acid (23-nor-5*β*-cholanic acid-3*α*,12*α*-diol) as an internal standard.

The 12*α*OH BA measured in this experiment were as follows: CA (5*β*-cholanic acid-3*α*,7*α*,12*α*-triol), DCA (5*β*-cholanic acid-3*α*,12*α*-diol), ursocholic acid (5*β*-cholanic acid-3*α*,7*β*,12*α*-triol), taurocholic acid (5*β*-cholanic acid-3*α*,7*α*,12*α*-triol-*N*-(2-sulfoethyl)-amide), taurodeoxycholic acid (5*α*-cholanic acid-3*α*,12*α*-diol-*N*-(2-sulfoethyl)-amide), glycocholic acid (5*β*-cholanic acid-3*α*,7*α*,12*α*-triol-*N*-(carboxymethyl)-amide), glycodeoxycholic acid (5*β*-cholanic acid-3*α*,12*α*-diol-*N*-(carboxymethyl)-amide), 7-oxo-deoxycholic acid (5*β*-cholanic acid-3*α*,12*α*-diol-7-one), 12-oxo-lithocholic acid (12oLCA; 5*β*-cholanic acid-3*α*-ol-12-one) and 5*β*-cholanic acid-12*α*-ol-3-one (3o12*α*).

The non-12*α*OH BA measured in this experiment were as follows: *α*-muricholic acid (5*β*-cholanic acid-3*α*,6*β*,7*α*-triol), *β*-muricholic acid (5*β*-cholanic acid-3*α*,6*β*,7*β*-triol), ω-muricholic acid (5*β*-cholanic acid-3*α*,6*α*,7*β*-triol), chenodeoxycholic acid (5*β*-cholanic acid-3*α*,7*α*-diol), hyocholic acid (5*β*-cholanic acid-3*α*,6*α*,7*α*-triol), hyodeoxycholic acid (5*β*-cholanic acid-3*α*,6*α*-diol), ursodeoxycholic acid (5*β*-cholanic acid-3*α*,7*β*-diol), lithocholic acid (LCA; 5*β*-cholanic acid-3*α*-ol), tauro-*α*-muricholic acid (5*β*-cholanic acid-3*α*,6*β*,7*α*-triol-*N*-(2-sulfoethyl)-amide), tauro-*β*-muricholic acid (5*β*-cholanic acid-3*α*,6*β*,7*β*-triol-*N*-(2-sulfoethyl)-amide), tauro-ω-muricholic acid (5*β*-cholanic acid-3*α*,6*α*,7*β*-triol-*N*-(2-sulfoethyl)-amide), taurochenodeoxycholic acid (5*β*-cholanic acid-3*α*,7*α*-diol-*N*-(2-sulfoethyl)-amide), taurohyodeoxycholic acid (5*β*-cholanic acid-3*α*,6*α*-diol-*N*-(2-sulfoethyl)-amide), taurolithocholic acid (5*β*-cholanic acid-3*α*-ol-*N*-(2-sulfoethyl)-amide), tauroursodeoxycholic acid (5*β*-cholanic acid-3*α*,7*β*-diol-*N*-(2-sulfoethyl)-amide), glycochenodeoxycholic acid (5*β*-cholanic acid-3*α*,7*α*-diol-*N*-(carboxymethyl)-amide), glycohyodeoxycholic acid (5*β*-cholanic acid-3*α*,6*α*-diol-*N*-(carboxymethyl)-amide), glycoursodeoxycholic acid (5*β*-cholanic acid-3*α*,7*β*-diol-*N*-(carboxymethyl)-amide), glycolithocholic acid (5*α*-cholanic acid-3*α*-ol-*N*-(carboxymethyl)-amide) and 7-oxo-lithocholic acid (5*β*-cholanic acid-3*α*-ol-7-one).

All BA, except for ursocholic acid, were purchased from Steraloids Inc. Ursocholic acid was obtained from Toronto Research Chemicals.

Real-time quantitative PCR

mRNA expression was measured in the liver and ileal mucosa using real-time quantitative PCR[23]. Total RNA was extracted using an RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions, and the RNA concentration was measured using a NanoDrop™ Lite Spectrophotometer (Thermo Scientific). Complementary DNA was synthesised from 1 μg of RNA using ReverTraAce® quantitative PCR RT master mix with gDNA remover (Toyobo Co. Ltd), according to the manufacturer's instructions. Quantitative PCR was performed on an Mx3000P™ Real-Time PCR system (Stratagene) using the SYBR® Green method or TaqMan® method, as described previously[23]. The SYBR® Green method was performed with specific primer pairs for ribosomal protein lateral stalk subunit P0 (*Rplp0*, forward: 5′-GGCAAGAACACCATGATGCG-3′; reverse: 5′-GTGATGCCCAAAGCTTGGAA-3′, 5 μM), apical Na-dependent bile acid transporter (*Asbt*, *SLC10A2*; forward: 5′-GGTGACATGGACCTCAGTGTT-3′; reverse: 5′-GTAGGGGATCACAATCGTTCCT-3′, 10 μM), ATP-binding cassette subfamily C member 2 (*Abcc2*, *MRP2*; forward: 5′-GCTGGTTGGAAACTTGGTCG-3′; reverse: 5′-CAGGGCGCCTCATTCTCTAC-3′, 10 μM), organic solute transporter subunit *α* (*Osta*, *SLC51A*; forward: 5′-CCCTCATACTTACCAGGAAGAAGCTAC-3′; reverse: 5′-CCATCAGGAATGAGAAACAGGC-3′, 10 μM) and organic solute transporter subunit *β* (*Ostb*, *SLC51B*; forward: 5′-TATTCCATCCTGGTTCTGGCAGT-3′; reverse: 5′-CGTTGTCTTGTGGCTGCTTCTT-3′, 10 μM). Relative expression levels were calculated for each sample after normalisation to *Rplp0* as a reference gene using the standard curve method. The TaqMan® Gene Expression Assays (Thermo Fisher Scientific) used in this study were as follows: Rn03302271_gH for RPLP0, Rn01495769_m1 for sterol regulatory element binding protein-1 (*Srebp1*), Rn01463550_m1 for fatty acid synthase (*Fasn*), Rn04181355_m1 for cell death-inducing DNA fragmentation factor-*α*-like effector A (*Cidea*) and Rn00580702_m1 for carnitine *O*-palmitoyltransferase 1 (*Cpt1*). Relative expression levels of these target mRNAs were calculated for each sample, as shown in the SYBR® Green method.

Organic acid analysis

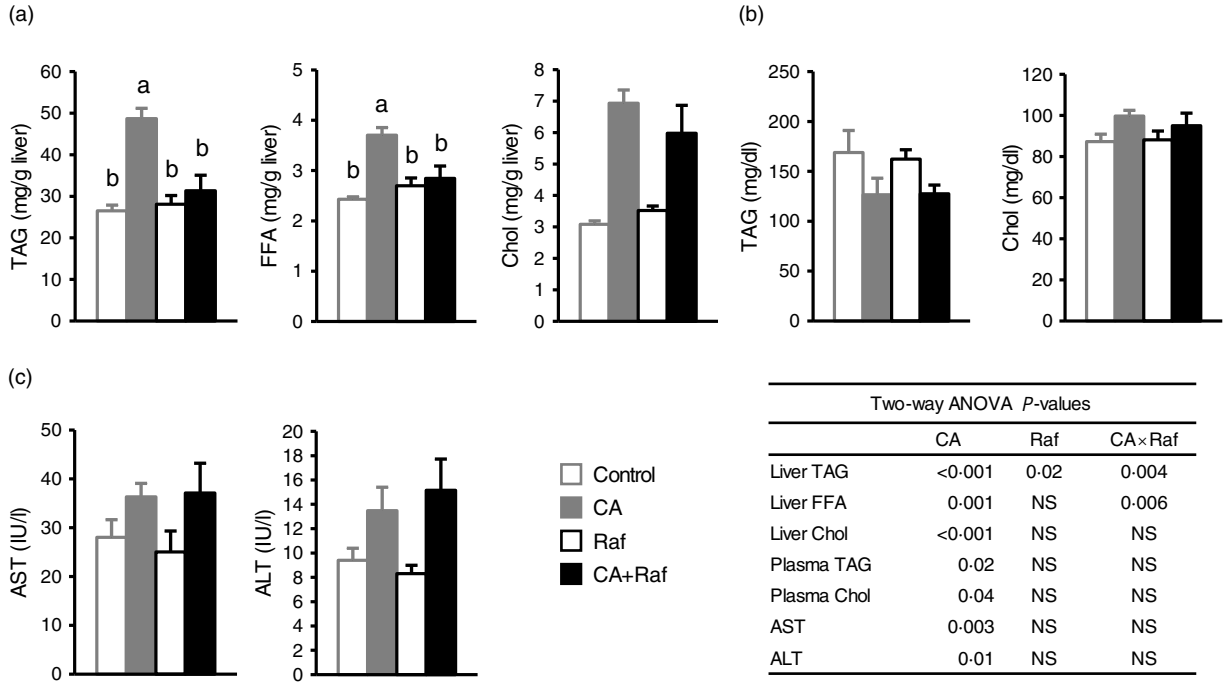
Organic acids in the caecal contents were measured using HPLC (Shimadzu) with crotonic acid (Wako Pure Chemical Industries) as an internal standard, according to the method of Hoshi[27]. The caecal contents were homogenised and neutralised with sodium hydroxide to prevent the extraction of SCFA. Fat-soluble substances in the supernatant were removed using chloroform, and the aqueous phase was passed through a membrane filter (cellulose acetate, 0·20 μm pore size; DISMIC-13cp; Toyo Roshi Kaisha). The samples were analysed using an HPLC system (Shimadzu). The equipment of the HPLC system consisted of LC-20AD pump, SIL-20AC auto-sampler and CDD-6A electro-conductivity detector. The mobile phase was 5 mmol/l *p*-toluenesulfonic acid, while the detection solution was 5 mmol/l *p*-toluenesulfonic acid containing 100 μmol/l EDTA and 20 mmol/l bis-tris.

Bomb calorimeter analysis

The faecal energy content was determined by combustion of approximately 0·3 g of dry faeces in a CA-4PJ automatic adiabatic bomb calorimeter (Shimadzu).

Statistical analyses

To determine an adequate sample size to identify significant differences in hepatic TAG concentration, a power analysis based on the experimental design was performed using G*Power (version 3.1.9.7)[28]. Based on an α probability of 0·05 and a power of 0·80, the effect size was estimated using the variance by special effects and error variances in an experiment (n 10 per group)[29] for hepatic TAG concentration. The



Two-way ANOVA *P*-values			
	CA	Raf	CA×Raf
Liver TAG	<0·001	0·02	0·004
Liver FFA	0·001	NS	0·006
Liver Chol	<0·001	NS	NS
Plasma TAG	0·02	NS	NS
Plasma Chol	0·04	NS	NS
AST	0·003	NS	NS
ALT	0·01	NS	NS

Fig. 1. Liver lipid and plasma parameters of rats fed control, cholic acid (CA), raffinose (Raf) or CA + Raf diet for 4 weeks. Liver lipids have been shown as (a) liver TAG, NEFA and cholesterol (Chol) concentrations. Plasma levels of (b) TAG, Chol and (c) transaminase activities (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)). Parameters of rats fed control and CA diets have been shown using blank and filled symbols, respectively. Parameters of rats fed diets with and without Raf supplementation have been shown in black and grey, respectively. The values have been expressed as mean values with their standard errors (*n* 5–6). *P*-values of two-way ANOVA for CA and Raf have been shown in the inset tables. Different superscript letters represent significant differences among the groups ($P < 0{\cdot}05$), obtained using Tukey–Kramer's test.

required sample size per group was determined to be 4–10 per group. All data have been presented as mean values with their standard errors. All the data were evaluated for normality and homogeneity of variance using the Shapiro–Wilk and Brown–Forsythe tests, respectively. The data were log-transformed to improve the homogeneity of the variance before ANOVA if necessary. Two-way ANOVA was used to evaluate significant interaction between the two factors, CA and Raf. Tukey–Kramer test was applied as a *post-hoc* test if a significant interaction was observed (CA × Raf). Multiple regression analysis was performed to identify predictors in parameters with significant differences in main effects of two-way ANOVA. Statistical analysis was performed using JMP version 14.0 (SAS Institute Inc.).

Results

To confirm whether Raf ingestion improves CA-induced steatosis and hepatic injury, we analysed parameters involved in hepatic steatosis. As shown in our previous study[10], CA feeding induced hepatic lipid accumulation (Fig. 1(a)) and increased plasma Chol, aspartate aminotransferase and alanine aminotransferase levels (Fig. 1(b) and (c)). In contrast, a reduction in plasma TAG was observed in the 4-week experiment (Fig. 1(b)). Notably, Raf ingestion normalised hepatic TAG and FFA concentrations in the CA-supplementation group (Fig. 1(a)). On the other hand, Raf diet did not affect liver Chol or plasma parameters (TAG, Chol and transaminase activities) (Fig. 1(a)–(c)). There were no significant differences in terms of total food intake, final body weights and epididymal adipose tissue weights between the groups (Table 2).

Thirty molecular species of BA in the liver, ileal contents, blood plasma and faeces were analysed using LC-MS at the end of the experimental period (Fig. 2). CA supplementation increased the concentrations of taurocholic acid and taurodeoxycholic acid in the liver. Two-way ANOVA revealed an interaction between CA and Raf in determination of the hepatic taurocholic acid concentration (Fig. 2(a)). The same was however not observed in the ileal contents (Fig. 2(b)). In case of portal and aortic plasma, alterations in CA concentrations were similar to those observed in the liver (Fig. 2(c) and (d)). CA supplementation increased the faecal DCA concentration, but a subtle influence of Raf diet was found on the composition of 12oLCA and 3o12α (Fig. 2(e)). There was an increase in faecal wet weight per day upon Raf supplementation (Table 2).

We also determined 12αOH BA in the liver, ileal contents, plasma and faeces (Fig. 2(f)). Ingestion of Raf diet partially reduced the 12αOH BA concentration in the livers of CA-fed rats. On the other hand, a high level of 12αOH BA was observed in the ileal contents of the CA + Raf-fed rats. The compositions of 12αOH BA in the portal and aortic plasmas were similar to those in the liver. In case of faecal BA, the increase in 12αOH BA upon CA supplementation was not altered upon further Raf supplementation. Excreted amount of 12αOH BA in the faeces was

British Journal of Nutrition

Table 2. Growth parameters and tissue weights in the rats fed the experimental diets for 4 weeks (Mean values with their standard errors)

	Two-way ANOVA								*P*-value		
	Control		CA		Raf		CA + Raf				
Control	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	CA	Raf	CA × Raf
Growth parameters (g)											
Total food intake	456	7·5	453	9·7	456	13	450	12	NS	NS	NS
Final body weight	256	7·2	256	6·2	261	4·2	253	4·2	NS	NS	NS
Tissue weight (g/100 g body weight)											
Liver weight	4·44	0·05	4·93	0·04	4·52	0·16	4·76	0·13	0·003	NS	NS
Epididymal adipose	2·05	0·11	1·92	0·16	2·24	0·12	2·00	0·1	NS	NS	NS
Other parameters											
Whole caecum (g)	3·97	0·27	4·16	0·09	6·11	0·37	5·82	0·68	NS	< 0·001	0·04
Caecal contents (g)	3·36	0·26	3·42	0·08	5·03	0·34	4·79	0·56	NS	< 0·001	NS
Caecal pH	7·25	0·13	7·37	0·30	6·85	0·12	6·82	0·10	NS	0·02	NS
Faeces (g/d)	1·31	0·11	1·39	0·08	1·89	0·21	1·64	0·11	NS	0·006	NS

tended to be increased as follows: 3·05 (SEM 0·50) μmol/d in control, 12·64 (SEM 1·25) μmol/d in CA, 3·43 (SEM 0·30) μmol/d in Raf and 15·14 (SEM 2·22) μmol/d in CA + Raf (two-way ANOVA *P*-values were < 0·0001 for CA, NS for Raf and NS for CA × Raf; NS). It is possible that an enhancement of 12*α*OH BA excretion in the urine although we have not measured in the present study. We consider that Raf supplementation enhanced faecal 12*α*OH BA excretion. We deduced a relationship between 12*α*OH BA and hepatic lipid accumulation and examined the association between 12*α*OH BA and liver TAG using Pearson's correlation analysis. The portal plasma 12*α*OH BA concentration correlated with the liver TAG concentration (Fig. 2(g); $R^2 = 0{\cdot}52$, $P < 0{\cdot}001$). Similar correlations of liver TAG concentration were found with the hepatic 12*α*OH BA concentration ($R^2 = 0{\cdot}49$, $P < 0{\cdot}001$) or aortic 12*α*OH BA concentration ($R^2 = 0{\cdot}47$, $P < 0{\cdot}001$).

Faecal lipids and faecal energy content were analysed. A significant increase in TAG was found upon Raf supplementation, while a reduction in faecal FFA was observed upon CA supplementation (Fig. 3(a)). Faecal energy excretion correlated with the faecal weight ($P < 0{\cdot}001$, $R^2 = 0{\cdot}91$). There was no difference in faecal Chol excretion upon the dietary interventions (Fig. 3(a)). Notably, Raf ingestion increased faecal energy excretion (Fig. 3(a)). We measured the levels of caecal organic acids to find an association between hepatic steatosis and caecal fermentation (Fig. 3(b)). Raf supplementation increased the concentrations of acetate and succinate (Fig. 3(b)), accompanied by a reduction in the caecal pH (Table 2). There was a significant increase in the weights of the whole caecum, caecal contents and faeces upon Raf ingestion (Table 2). We conducted multiple linear regression analysis to determine predictors of liver TAG and found that the concentrations of aortic 12*α*OH BA and caecal acetic acid could serve as predictors of hepatic steatosis (Fig. 3(c), inset table).

No significant differences were observed in the hepatic mRNA expression levels of *Srebp1*, *Fasn*, *CideA* and *Cpt1* (data not shown). In case of ileal mRNA expression of BA transporters responsible for enterohepatic BA circulation (Fig. 3), CA supplementation resulted in an increase in *Abcc2* expression. In addition, an interaction between CA and Raf was observed in *Asbt* expression, although no significant difference was observed in a *post hoc* test. There were no significant differences in the expression levels of *Osta* and *Ostb* in the ileal mucosa (Fig. 3).

Discussion

In this study, we investigated whether dietary Raf suppresses hepatic lipid accumulation induced by the CA supplementation to diet[10] based on a report that shows Raf modulates BA metabolism in mice fed a high-fat/western-style diet[15]. We successfully detected the CA-induced hepatic TAG accumulation in week 4 and also observed dietary Raf normalised hepatic TAG accumulation in the CA-fed rats accompanied by reduced 12*α*OH BA concentrations in liver and portal plasma.

In our CA-feeding hepatic steatosis model[10], the nutrient composition of the CA-supplemented diet was almost the same as that of the control diet, other than the addition of CA at 0·5 g/kg diet. In addition, there was no alteration in food consumption and body weight. Nevertheless, the CA diet induces hepatic steatosis without obesity and liver inflammation[10], which may represent an early stage of fatty liver. Such simple fatty liver without obesity is also observed in humans[30]. In order to find a way to prevent NAFLD, it is important to identify what happens at the onset of steatosis that can be easily modified with a simple dietary intervention. Based on this novel fatty liver model, we investigated whether the use of oligosaccharides that affect BA metabolism leads to the prevention of hepatic steatosis. The present study showed that dietary raffinose reduced hepatic lipid accumulation via suppression of enterohepatic 12*α*OH BA circulation.

Since *de novo* lipogenesis is an important event in CA-induced steatosis, we analysed mRNA expression of genes closely associated with *de novo* lipogenesis. However, no significant alteration was detected in the expression levels of *Srebp1* and *Fasn*, even in the livers of CA-fed rats. From another point of view, the involvement of dietary lipids is also of interest in steatosis. To ascertain a balance of dietary fat, we measured the faecal excretions of energy and lipids such as TAG, FFA and Chol (Fig. 3). There was no difference in faecal energy

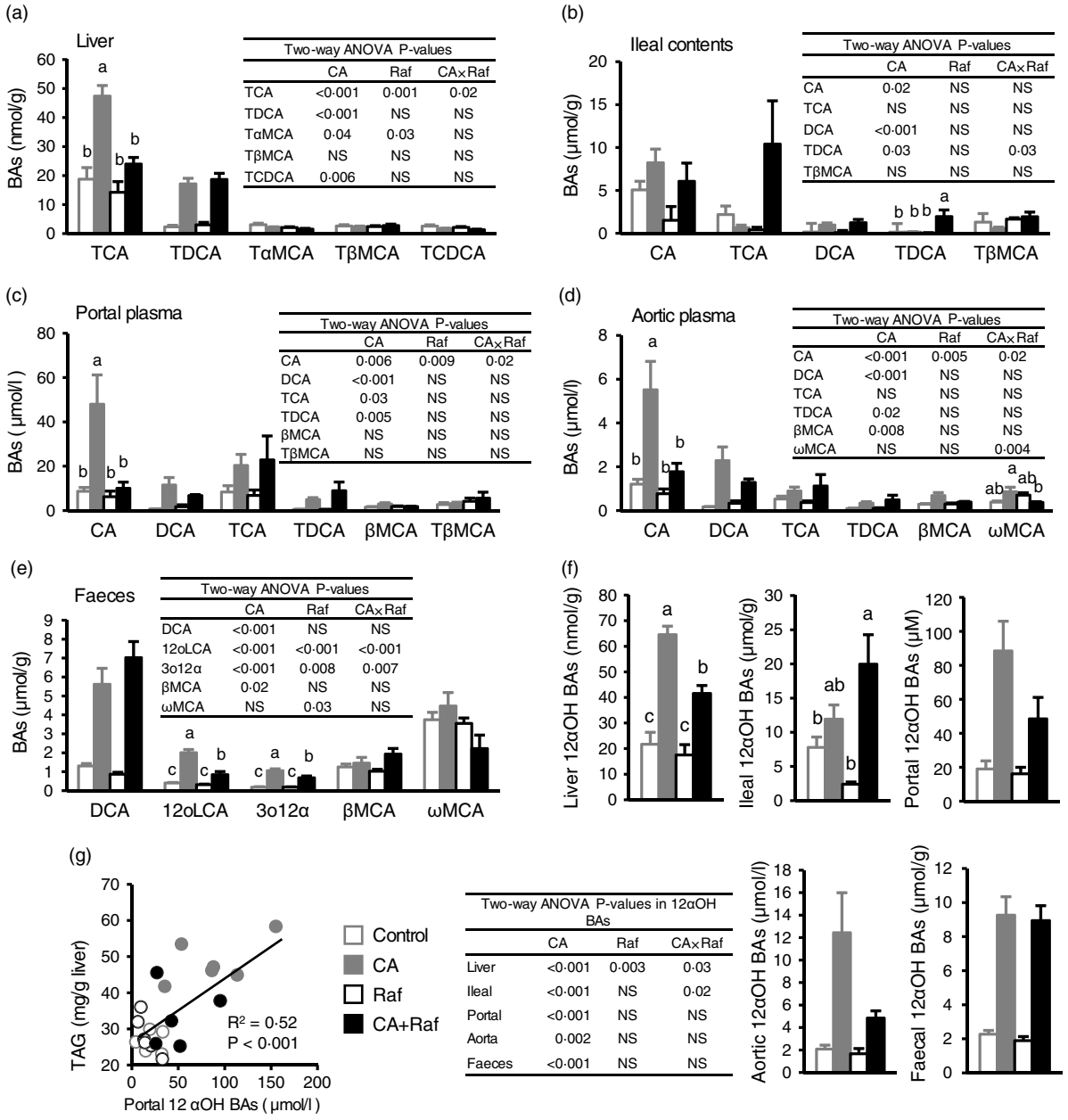
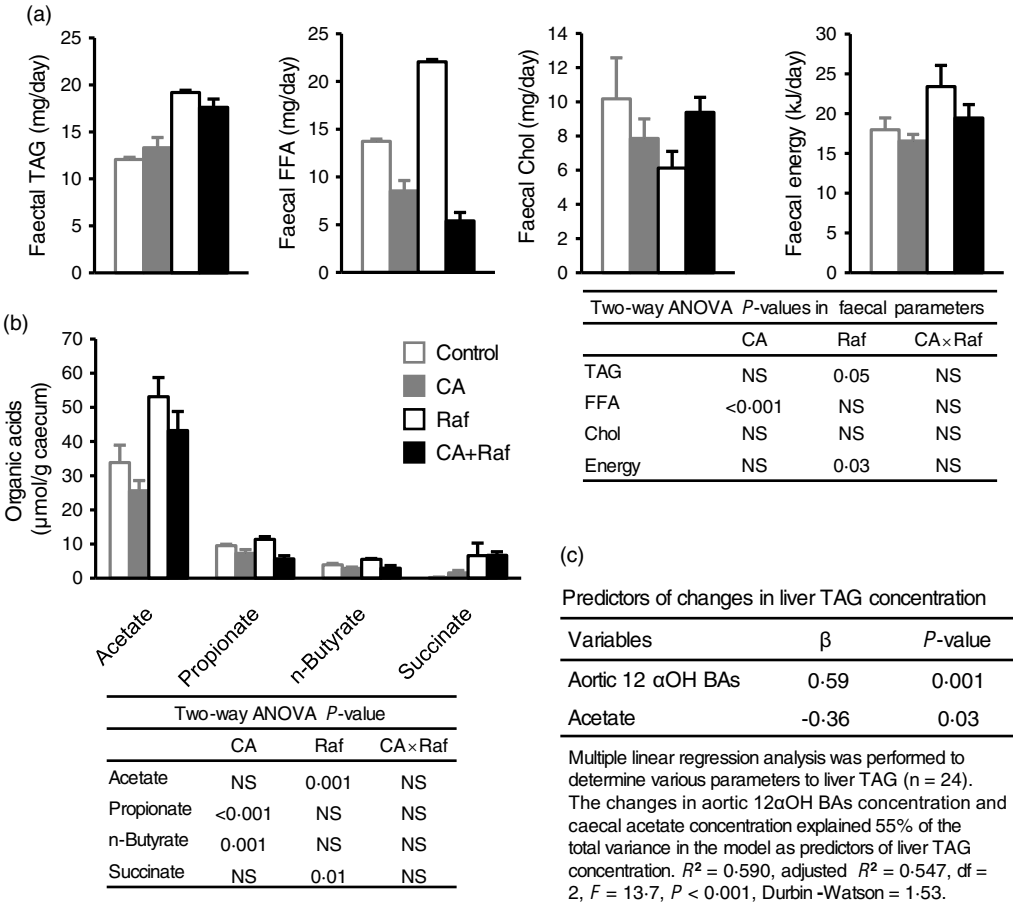


Fig. 2. Bile acid (BA) composition in samples from rats fed control, cholic acid (CA), raffinose (Raf) or CA + Raf diet for 4 weeks. Concentration of each BA molecule in (a) liver, (b) ileal contents, (c) portal plasma, (d) aortic plasma and (e) faeces. Concentrations of 12α-hydroxylated (12αOH) BA in (f) liver, ileal contents, portal plasma, aortic plasma and faeces. (g) Pearson's correlation between the concentrations of portal 12αOH BA and liver TAG. Parameters of rats fed control and CA diets have been shown using blank and filled symbols, respectively. Parameters of rats fed diets with and without Raf supplementation have been shown in black and grey, respectively. The values have been expressed as mean values with their standard errors (n 5–6). P-values of two-way ANOVA for CA and Raf have been shown in the inset tables. Different superscript letters represent significant differences among the groups ($P < 0{\cdot}05$), obtained using Tukey–Kramer's test.

excretion between the control and CA diet groups, which suggests that the retention of dietary fat is almost comparable between the groups. On the other hand, enhanced faecal energy excretion upon supplementation with dietary Raf is another possible explanation for the partial amelioration of dyslipidaemia by Raf. However, in the present study, this effect may not contribute to hepatic lipid normalisation by dietary Raf in the CA-fed condition because alteration of Raf-induced energy excretion was not consistent with hepatic TAG accumulation and simply correlated with faecal weight. Both *de novo* lipogenesis and dietary fat were not clearly associated with changes in hepatic TAG concentration in this study, which is probably due to the short term of the experiments, which makes it difficult to detect some subtle effects sharply. In other words, 12αOH BA metabolism is a sensitive parameter that reflects hepatic TAG concentration.

Modulation of enterohepatic 12αOH BA circulation is presumably a key event in Raf-mediated prevention of CA-induced hepatic steatosis. Raf feeding significantly reduced the portal CA concentration, while increasing the taurocholic acid concentration in ileal contents, especially under the CA-fed condition,

British Journal of Nutrition



Two-way ANOVA *P*-values in faecal parameters

	CA	Raf	CA×Raf
TAG	NS	0·05	NS
FFA	<0·001	NS	NS
Chol	NS	NS	NS
Energy	NS	0·03	NS

Two-way ANOVA *P*-value

	CA	Raf	CA×Raf
Acetate	NS	0·001	NS
Propionate	<0·001	NS	NS
n-Butyrate	0·001	NS	NS
Succinate	NS	0·01	NS

(c)

Predictors of changes in liver TAG concentration

Variables	β	*P*-value
Aortic 12 αOH BAs	0·59	0·001
Acetate	-0·36	0·03

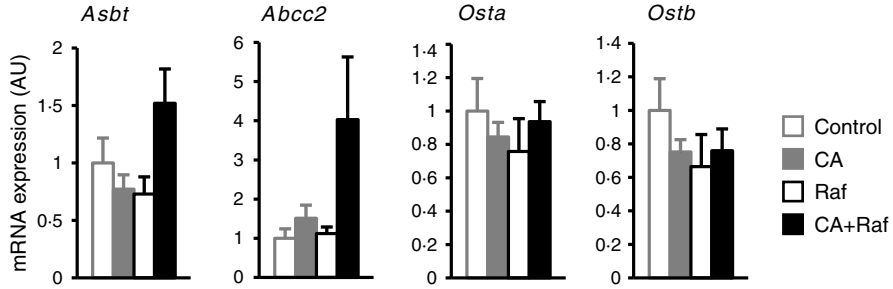
Multiple linear regression analysis was performed to determine various parameters to liver TAG (n = 24). The changes in aortic 12αOH BAs concentration and caecal acetate concentration explained 55% of the total variance in the model as predictors of liver TAG concentration. R^2 = 0·590, adjusted R^2 = 0·547, df = 2, F = 13·7, P < 0·001, Durbin-Watson = 1·53.

Fig. 3. Faecal lipid excretion, energy content, caecal parameters and multiple regression analysis of rats fed control, cholic acid (CA), raffinose (Raf) or CA + Raf diets for 4 weeks. (a) Faecal excretions of TAG, NEFA, cholesterol (Chol) and energy content per d were calculated from faecal weight collected for 24 h at the end of the experiment. (b) Organic acid concentrations. (c) Predictors of liver TAG concentration. Parameters in rats fed control and CA diets have been shown using blank and filled symbols, respectively. Parameters in rats fed diets with and without Raf supplementation have been shown in black and grey, respectively. The values have been expressed as mean values with their standard errors (*n* 5–6). *P*-values of two-way ANOVA for CA and Raf have been shown in the inset table.

which suggests suppression of enterohepatic 12*α*OH BA circulation. In addition, 12*α*OH BA in the portal blood was associated with liver TAG concentration. Enterohepatic BA circulation is mediated by ileal BA transporters[31]. ASBT is the major transporter in ileal epithelial cells that incorporates BA from the luminal contents, while ABCC2 is another transporter that secretes BA from the enterocyte to the lumen[32]. Incorporated BA are secreted from the enterocytes to the portal blood via organic solute transporter subunit *α* and organic solute transporter subunit *β*[32]. Increased ABCC2 gene expression may contribute to BA excretion to the lumen, which contributes to the suppression of enterohepatic 12*α*OH BA circulation. Such reduction of enterohepatic 12*α*OH BA circulation is not necessarily observed in other types of dietary oligosaccharides. Dietary difructose anhydride III does not lower the portal 12*α*OH BA concentration in CA-fed rats under similar experimental conditions[10]. In the literature, Chol-enriched diet triggers down-regulation of ASBT expression, a reduction in ileal BA uptake and an increase in the faecal excretion of BA in mice[33]. Another study showed that a decrease in luminal BA induces up-regulation of ASBT expression and promotes uptake of ileal BA, which results in reduction of BA excretion in ampicillin-administered mice[34]. These studies suggest that ASBT expression depends on the luminal BA environment in order to maintain BA homoeostasis in the body. In this experiment, a significant interaction was observed between CA and Raf supplementation in case of *Asbt* expression. These observations suggest that suppression of enterohepatic 12*α*OH BA circulation plays a preventive role in the development of hepatic TAG accumulation.

Raf feeding increased the levels of acetic acid (Fig. 3) and lowered the caecal pH (Table 2). In addition, acetic acid and aortic 12*α*OH BA were predictors of liver TAG concentration (Fig. 3). Oral administration with acetate has been shown to improve hepatic steatosis in Otsuka Long-Evans Tokushima Fatty rats, accompanied by an increase in the AMP:ATP ratio, which contribute to the promotion of AMP-activated protein kinase and reduction in fatty acid synthesis[35]. Although such an effect was expected in the present study, a clear influence on gene expression of the hepatic *de novo* lipogenesis-related genes was not identified (Fig. 4).

Acidification of the intestine by ingestion of difructose anhydride III alters the gut microbiota and suppresses secondary BA formation[36]. On the other hand, DCA production was not inhibited by dietary Raf in the CA-fed rats in the present study



Two-way ANOVA *P*-value			
	CA	Raf	CA×Raf
Asbt	NS	NS	0·03
Abcc2	0·04	NS	NS
Osta	NS	NS	NS
Ostb	NS	NS	NS

Fig. 4. Ileal gene expression in rats fed control, cholic acid (CA), raffinose (Raf) or CA + Raf diet for 4 weeks. Ileal mRNA expression levels of apical Na-dependent bile acid transporter (*Asbt*), ATP-binding cassette subfamily C member 2 (*Abcc2*), organic solute transporter subunit α (*Osta*) and organic solute transporter subunit β (*Ostb*). The data were normalised with ribosomal protein lateral stalk subunit P0 (*Rplp0*) mRNA expression. Parameters of rats fed control and CA diets have been shown using blank and filled symbols, respectively. Parameters of rats fed diets with and without Raf supplementation have been shown in black and grey, respectively. The values have been expressed as mean values with their standard errors (n 5–6). *P*-values of the two-way ANOVA for CA and Raf are shown in the inset table.

regardless of low pH environment in the caecal contents (Fig. 2(e)). *Clostridium* prefers a high pH of 6·5–7·5 in a continuous culture study[37], and 7α-dehydroxylase displays optimal activity at pH 7–8, while getting inhibited at a pH < 6·5 *in vitro*[38]. Raf lowered the caecal pH, but not to below pH 6·5. Hence, it is possible that upon Raf supplementation, *clostridium* could survive and the 7α-dehydroxylase activity was maintained, which enabled the conversion of primary BA into secondary BA. Stable DCA production upon CA supplementation may increase plasma transaminases, regardless of Raf supplementation in the present study. In our previous study[10], CA diet increased blood transaminase activities, which positively correlated with the DCA concentrations in the caecal contents in a long-term experiment. A study aimed to compare BA hepatotoxicity among CA, DCA and LCA showed that DCA supplementation associated with oxidative stress is the most hepatotoxic of the three[39]. In the present study, we expected that Raf supplementation would lead to altered gut microbiota and reduced secondary BA production, including DCA. However, the faecal DCA and liver taurodeoxycholic acid concentrations remained high under the CA-supplemented condition, regardless of Raf ingestion. Although Raf reduced 12αOH BA concentrations in the liver, the effect was partial and taurodeoxycholic acid remained at a high level upon CA supplementation. These observations suggest that DCA produced in the large intestine can be absorbed, which may be responsible for the increase in transaminase activities. Since a synbiotic combination in the diet reduces DCA production[40], synbiotics with Raf and appropriate bacteria can suppress secondary BA production. In the multiple hit hypothesis in liver disease[41], an additional second hit with oxidative stress or endoplasmic reticulum stress is necessary for disease progression. Inhibition of DCA production may reduce these risks, thus preventing further progression of fatty liver.

There were several limitations associated with the present study. First, we do not confirm that an increase in 12αOH in enterohepatic circulation directly induces hepatic lipid accumulation in humans. Second, the effect of dietary raffinose in humans remains obscure because intestinal bacteria that assimilate raffinose are not exactly the same as those in rats. Third, we have not clarified the mechanism by which 12αOH induces hepatic TAG accumulation in the CA-fed rat model. Further studies are necessary to address these issues. Fourth, there might be underpowered in some parameters even within the required range in the power analysis because the determination of the sample size was calculated for hepatic TAG concentration.

In conclusion, we found that normalisation of elevated 12αOH BA enterohepatic circulation by dietary Raf is associated with the amelioration of hepatic TAG accumulation induced upon CA supplementation. The present study also suggests faecal 12αOH BA as a marker for hepatic lipid accumulation.

Acknowledgements

This study was supported in part by the Japan Society for the Promotion of Science KAKENHI (S. I., grant numbers 19H2900 and 16K14917), the Japan Science and Technology Agency Centre of Innovation (S. I., grant number JPMJCE1301) and Nippon Beet Sugar Manufacturing Co. Ltd..

The authors' contributions are as follows: S. I., S. F. and A. Y. contributed to the study design and interpretation of the findings; K. M., H. K., K. U. and S. I. carried out the experiments and collected the data; K. M. and S. I. wrote the manuscript. All the authors have read and approved the final version of the manuscript.

The authors declare the following financial interest/relationship which may be considered as potential competing interests: S. I. and A. Y. received grants and were provided raffinose from Nippon Beet Sugar Manufacturing Co. Ltd. The company was not involved in this study.

References

1. Chalasani N, Younossi Z, Lavine JE, *et al.* (2012) The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* **55**, 2005–2023.
2. Younossi ZM, Golabi P, de Avila L, *et al.* (2019) The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: a systematic review and meta-analysis. *J Hepatol* **71**, 793–801.

3. Eguchi Y, Hyogo H, Ono M, *et al.* (2012) Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. *J Gastroenterol* **47**, 586–595.
4. Younossi ZM, Koenig AB, Abdelatif D, *et al.* (2016) Global epidemiology of nonalcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64**, 73–84.
5. Younossi Z, Stepanova M, Ong JP, *et al.* (2019) Nonalcoholic steatohepatitis is the fastest growing cause of hepatocellular carcinoma in liver transplant candidates. *Clin Gastroenterol Hepatol* **17**, 748–755.
6. Yoshitsugu R, Kikuchi K, Iwaya H, *et al.* (2019) Alteration of bile acid metabolism by a high-fat diet is associated with plasma transaminase activities and glucose intolerance in rats. *J Nutr Sci Vitaminol* **65**, 45–51.
7. Hori S, Abe T, Lee DG, *et al.* (2020) Association between 12α-hydroxylated bile acids and hepatic steatosis in rats fed a high-fat diet. *J Nutr Biochem* **83**, 108412.
8. Haeusler RA, Astiarraga B, Camastra S, *et al.* (2013) Human insulin resistance is associated with increased plasma levels of 12α-hydroxylated bile acids. *Diabetes* **62**, 4184–4191.
9. Uchida K, Nomura Y & Takeuchi N (1980) Effects of cholic acid, chenodeoxycholic acid, and their related bile acids on cholesterol, phospholipid, and bile acid levels in serum, liver, bile, and feces of rats. *J Biochem* **87**, 187–194.
10. Lee JY, Shimizu H, Hagio M, *et al.* (2020) 12α-Hydroxylated bile acid induces hepatic steatosis with dysbiosis in rats. *Biochim Biophys Acta Mol Cell Biol Lipids* **1865**, 158811.
11. Dinoto A, Suksomcheep A, Ishizuka S, *et al.* (2006) Modulation of rat caecal microbiota by administration of raffinose and encapsulated *Bifidobacterium breve*. *Appl Environ Microbiol* **72**, 784–792.
12. Hayakawa S & Hattori T (1970) 7α-Dehydroxylation of cholic acid by *Clostridium bifermentans* strain ATCC 9714 and *Clostridium sordellii* strain NCIB 6929. *FEBS Lett* **6**, 131–133.
13. Garcia-Diez F, Garcia-Mediavilla V, Bayon JE, *et al.* (1996) Pectin feeding influences fecal bile acid excretion, hepatic bile acid and cholesterol synthesis and serum cholesterol in rats. *J Nutr* **126**, 1766–1771.
14. Hoving LR, Katiraei S, Heijink M, *et al.* Dietary mannan oligosaccharides modulate gut microbiota, increase fecal bile acid excretion, and decrease plasma cholesterol and atherosclerosis development. *Mol Nutr Food Res* **62**, 1700942.
15. Dai Z, Feng S, Liu AB, *et al.* (2019) Protective effects of α-galacto-oligosaccharides against a high-fat/western-style diet-induced metabolic abnormalities in mice. *Food Funct* **10**, 3660–3670.
16. Reeves PG, Nielsen FH & Fahey GC (1993) AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition Writing Committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.
17. Hashimoto N, Matsui I, Ishizuka S, *et al.* (2020) Lithocholic acid increases intestinal phosphate and calcium absorption in a vitamin D receptor dependent but transcellular pathway independent manner. *Kidney Int* **97**, 1164–1180.
18. Bernstein H, Bernstein C, Payne CM, *et al.* (2005) Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* **589**, 47–65.
19. Kurdi P, Kawanishi K, Mizutani K, *et al.* (2006) Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. *J Bacteriol* **188**, 1979–1986.
20. Islam KBMS, Fukiya S, Hagio M, *et al.* (2011) Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* **141**, 1773–1781.
21. Ishizuka S, Iwama A, Dinoto A, *et al.* (2009) Synbiotic promotion of epithelial proliferation by orally ingested encapsulated *Bifidobacterium breve* and raffinose in the small intestine of rats. *Mol Nutr Food Res* **53**, S62–S67.
22. Janzi S, Dias JA, Martinsson A, *et al.* (2020) Association between dietary fiber intake and risk of incident aortic stenosis. *Nutr Metab Cardiovasc Dis* **30**, 2180–2185.
23. Hori S, Hara H & Ishizuka S (2018) Marginal iron deficiency enhances liver triglyceride accumulation in rats fed a high-sucrose diet. *Biosci Biotechnol Biochem* **82**, 2140–2148.
24. Hagio M, Matsumoto M, Fukushima M, *et al.* (2009) Improved analysis of bile acids in tissues and intestinal contents of rats using LC/ESI-MS. *J Lipid Res* **50**, 173–180.
25. Hagio M, Matsumoto M & Ishizuka S (2011) Bile acid analysis in various biological samples using ultra performance liquid chromatography/electrospray ionization-mass spectrometry (UPLC/ESI-MS). *Methods Mol Biol* **708**, 119–129.
26. Lee DG, Hori S, Kohmoto O, *et al.* (2019) Ingestion of difructose anhydride III partially suppresses the deconjugation and 7α-dehydroxylation of bile acids in rats fed with a cholic acid-supplemented diet. *Biosci Biotechnol Biochem* **83**, 1329–1335.
27. Hoshi S, Sakata T, Mikuni K, *et al.* (1994) Galactosylsucrose and xylosylfructoside alter digestive tract size and concentrations of caecal organic acids in rats fed diets containing cholesterol and cholic acid. *J Nutr* **124**, 52–60.
28. Faul F, Erdfelder E, Lang AG, *et al.* (2007) G × Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* **39**, 175–191.
29. Shimoda T, Hori S, Maegawa K, *et al.* (2020) A low coefficient of variation in hepatic triglyceride concentration in an inbred rat strain. *Lipids Health Dis* **19**, 137.
30. Kojima S, Watanabe N, Numata M, *et al.* (2003) Increase in the prevalence of fatty liver in Japan over the past 12 years: analysis of clinical background. *J Gastroenterol* **38**, 954–961.
31. Dawson PA, Haywood J, Craddock AL, *et al.* (2003) Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice. *J Biol Chem* **278**, 33920–33927.
32. Dawson PA & Karpen SJ (2015) Intestinal transport and metabolism of bile acids. *J Lipid Res* **56**, 1085–1099.
33. Thomas C, Landrier J-F, Gaillard D, *et al.* (2006) Cholesterol dependent downregulation of mouse and human apical sodium dependent bile acid transporter (ASBT) gene expression: molecular mechanism and physiological consequences. *Gut* **55**, 1321–1331.
34. Miyata M, Yamakawa H, Hamatsu M, *et al.* (2011) Enterobacteria modulate intestinal bile acid transport and homeostasis through apical sodium-dependent bile acid transporter (SLC10A2) expression. *J Pharmacol Exp Ther* **336**, 188–196.
35. Yamashita H, Fujisawa K, Ito E, *et al.* (2007) Improvement of obesity and glucose tolerance by acetate in type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Biosci Biotechnol Biochem* **71**, 1236–1243.
36. Minamida K, Kaneko M, Ohashi M, *et al.* (2005) Effects of difructose anhydride III (DFA III) administration on bile acids and growth of DFA III-assimilating bacterium *Ruminococcus productus* on rat intestine. *J Biosci Bioeng* **99**, 548–554.
37. Edwards CA, Duerden BI & Read NW (1985) The effects of pH on colonic bacteria grown in continuous culture. *J Med Microbiol* **19**, 169–180.

British Journal of Nutrition

38. Macdonald IA, Singh G, Mahony DE, *et al.* (1978) Effect of pH on bile salt degradation by mixed fecal cultures. *Steroids* **32**, 245–256.
39. Delzenne NM, Calderon PB, Taper HS, *et al.* (1992) Comparative hepatotoxicity of cholic acid, deoxycholic acid and lithocholic acid in the rat: *in vivo* and *in vitro* studies. *Toxicol Lett* **61**, 291–304.
40. Lee Y, Yoshitsugu R, Kikuchi K, *et al.* (2016) Combination of soya pulp and *Bacillus coagulans* lilac-01 improves intestinal bile acid metabolism without impairing the effects of prebiotics in rats fed a cholic acid-supplemented diet. *Br J Nutr* **116**, 603–610.
41. Buzzetti E, Pinzani M & Tsochatzis EA (2016) The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* **65**, 1038–1048.

British Journal of Nutrition