Intestinal α1-2-Fucosylation Contributes to Obesity and Steatohepatitis in Mice

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SUMMARY
Intestinal α1-2-fucosylation mediates host–microbe interactions and can shape the metabolism of intestinal microorganisms. Here, we identified that mice lacking α1-2-fucosylation were protected from Western diet–induced features of obesity and steatohepatitis and this protection was mediated through the intestinal microbiota.

BACKGROUND & AIMS: Fucosyltransferase 2 (Fut2)-mediated intestinal α1-2-fucosylation is important for host–microbe interactions and has been associated with several diseases, but its role in obesity and hepatic steatohepatitis is not known. The aim of this study was to investigate the role of Fut2 in a Western-style diet–induced mouse model of obesity and steatohepatitis.

METHODS: Wild-type (WT) and Fut2-deficient littermate mice were used and features of the metabolic syndrome and steatohepatitis were assessed after 20 weeks of Western diet feeding.

RESULTS: Intestinal α1-2-fucosylation was suppressed in WT mice after Western diet feeding, and supplementation of α1-2-fucosylated glycans exacerbated obesity and steatohepatitis in these mice. Fut2-deficient mice were protected from Western diet–induced features of obesity and steatohepatitis despite an increased caloric intake. These mice have increased energy expenditure and thermogenesis, as evidenced by a higher core body temperature. Protection from obesity and steatohepatitis
associated with Fut2 deficiency is transmissible to WT mice via microbiota exchange; phenotypic differences between Western diet-fed WT and Fut2-deficient mice were reduced with antibiotic treatment. Fut2 deficiency attenuated diet-induced bile acid accumulation by altered relative abundance of bacterial enzyme 7-α-hydroxysteroid dehydrogenases metabolizing bile acids and by increased fecal excretion of secondary bile acids. This also was associated with increased intestinal farnesoid X receptor/fibroblast growth factor 15 signaling, which inhibits hepatic synthesis of bile acids. Dietary supplementation of α1-2-fucosylated glycans abrogates the protective effects of Fut2 deficiency.

CONCLUSIONS: α1-2-fucosylation is an important host-derived regulator of intestinal microbiota and plays an important role for the pathogenesis of obesity and steatohepatitis in mice. (Cell Mol Gastroenterol Hepatol 2021;12:293–320; https://doi.org/10.1016/j.jcmgh.2021.02.009)

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Worldwide obesity has nearly tripled since 1975, and more than 1.9 billion adults were overweight, and among them more than 650 million were obese in 2016. Nonalcoholic fatty liver disease (NAFLD), which is associated commonly with obesity, has become a leading cause of chronic liver disease and is one of the main causes for obesity-related deaths. Nonalcoholic steatohepatitis (NASH), characterized by hepatic steatosis with inflammation and fibrosis, is the most serious form of NAFLD and can progress to cirrhosis and hepatocellular carcinoma. The pathogenesis of obesity and NASH involves a complex interaction and cross-talk between environmental factors, host genetics, and intestinal microbiota.

The enzyme fucosyltransferase 2 (Fut2) encoded by the α1-2-fucosyltransferase 2 gene (Fut2) catalyzes the process of α1-2-fucosylation, which adds fucose to glycolipids and glycoproteins, as well as unconjugated glycans such as human milk oligosaccharides. In human beings and mice, Fut2 is expressed mainly in epithelial cells of the digestive (intestine and gallbladder) and genital tract, whereas it is absent in liver and adipose tissues. Fut2 is highly expressed in the distal gut where abundant symbiotic microbes are colonizing. Fucosylated glycans are important for host–microbe interactions. Membrane and secreted α1-2-linked fucose can be cleaved by bacterial fucosidase and the liberated L-fucose is used by certain bacteria. L-fucose can serve as substrate for bacteria for the synthesis of fucosylated polysaccharides, regulation of gene expression through the fucose operon, and undergoing catabolism for energy. Epithelial α1-2-fucosylation also can be regulated by microbes because germ-free mice have impaired α1-2-fucosylation in the intestine, which can be restored by colonization with commensal microbes. Systemic exposure to Toll-like receptor ligands induces rapid α1-2-fucosylation of epithelial cells in the small intestine. Intestinal α1-2-fucosylation has been implicated in the pathogenesis of several diseases that are associated with the intestinal microbiome, including Crohn’s disease, chronic pancreatitis, primary sclerosing cholangitis, and several infectious diseases. In this study, we investigated whether changes of intestinal α1-2-fucosylation affect Western diet–induced obesity and steatohepatitis in mice.

Results

Feeding a Western Diet Reduces Intestinal α1-2-Fucosylation in Mice

Fut2 is highly expressed in the distal intestinal tract (Figure 1A). Fut2 mediates α1-2-fucosylation of proteins and lipids on the surface of intestinal epithelial cells and the gallbladder (Figure 1B). Fut4 and Fut8 mediate α1-3- and α1-6-fucosylation, respectively, and also are expressed in mouse intestine, but to a lesser degree than Fut2. To evaluate the role of intestinal fucosylation for obesity and steatohepatitis, we first compared expression of Fut2, Fut4, and Fut8 genes and α1-2-, α1-3-, and α1-6-fucosylated glycans in ileum and colon, in control diet and in Western diet-fed wild-type (WT) mice. Consistent with previous studies, Fut2 was more abundant in the colon compared with the ileum in mice fed a control diet (Figure 2A). Both Fut2 messenger RNA (mRNA) and α1-2-fucosylated glycans were significantly lower after Western diet feeding for 20 weeks as evidenced by quantitative reverse-transcription polymerase chain reaction (PCR) and immunohistochemistry staining (Figure 2A and D). Although Fut8 mRNA was up-regulated in colon tissue after Western diet feeding (Figure 2C), expression of colonic α1-6-fucosylated glycans was not changed (Figure 2D). Fut4 mRNA and α1-3-fucosylated glycans were not changed in colons of mice fed a Western diet (Figure 2B and D). These results indicate that colonic Fut2-mediated α1-2-fucosylation is reduced in a Western diet–induced obesity and steatohepatitis mouse model.

To restore intestinal α1-2-fucosylation, we supplemented WT mice with 2′-fucosyllactose (2′-FL) in the drinking water. 2′-FL is a prebiotic commonly found in human milk that cannot be used by the host. Intestinal bacteria can cleave 2′-FL and release L-fucose. Supplementation of 2′-FL resulted in increased body and liver weight, more liver...
injury (as evidenced by higher alanine aminotransferase [ALT] levels), and hepatic steatosis in Western diet–fed but not control diet–fed mice (Figure 2E). This raises the possibility that the down-regulation of α1-2-fucosylation in Western diet–fed mice is a protective mechanism.

**Fut2-Deficient Mice Are Protected From Western Diet–Induced Obesity and Metabolic Syndrome**

To further study the role of α1-2-fucosylation for pathogenesis of diet-induced obesity and steatohepatitis, Fut2−/− and WT littermate mice were subjected to feeding of a Western diet for 20 weeks. We confirmed that Fut2−/− mice lacked expression of α1-2-fucosylated glycans in the intestine by immunohistochemistry staining (Figure 3). Fut2−/− mice gained significantly less body weight compared with WT mice (Figure 4A). Fut2 deficiency did not affect epididymal white adipose tissue weight or brown adipose tissue weight (Figure 5A). Fut2−/− mice showed improved metabolic and endocrine profiles including increased insulin sensitivity and lower plasma levels of cholesterol and leptin compared with WT mice after a Western diet (Figure 4B–D). We noticed that Western diet–fed Fut2−/− mice had a significantly higher caloric intake than WT littermate mice (Figure 4E). Therefore, we restricted the total caloric intake of Fut2−/− mice to make it equal to the caloric intake of WT mice during Western diet feeding for 20 weeks. Calorie-restricted Fut2−/− mice were fully protected from features of the metabolic syndrome as evidenced by lower body weight and brown adipose tissue, increased insulin sensitivity, and lower levels of plasma cholesterol and leptin than Fut2−/− mice with unrestricted access to a Western diet (Figures 4A–D and 5A).

There was no difference in fecal lipid content during Western diet feeding, indicating that Fut2−/− mice have similar levels of intestinal lipid absorption (Figure 5B). We compared the metabolic rates of WT and Fut2−/− mice on different diets, and no difference was found in control......
diet-fed mice. In Western diet-fed mice, oxygen consumption (VO2) and carbon dioxide production (VCO2) rate were slightly higher in Fut2-/- compared with WT mice (Figure 6A). Western diet-fed Fut2-/- mice had a higher respiratory exchange ratio, energy expenditure, and more vertical activity compared with WT mice (Figures 4F and 6A). These differences were more obvious during the dark cycles (Figure 6A) compared with the light cycles (Figure 6B), which is consistent with increased nocturnal activity of mice. In line with increased energy expenditure, Western diet-fed Fut2-/- mice generated more heat, with a significantly higher core body temperature (Figure 4G). An increased protein level of uncoupling protein 1 (Ucp1) in brown adipose tissue (Figure 4H) indicates augmented nonshivering thermogenesis in Western diet-fed Fut2-/- mice compared with WT mice. Taken together, Fut2 deficiency increases energy expenditure and thermogenesis in brown adipose tissue, which might contribute to protection from Western diet-induced obesity.

**Fut2 Deficiency Attenuates Western Diet–Induced Steatohepatitis**

To assess the role of Fut2 for the development of steatohepatitis, we investigated parameters of liver injury, steatosis, inflammation, and fibrosis. Western diet-induced liver injury as assessed by levels of ALT (Figure 7A) and hepatic steatosis as evaluated by liver weight, hepatic triglycerides, and H&E staining (Figure 7B and C) were lower in Western diet-fed Fut2-/- mice compared with WT mice. Hepatic expression of inflammatory genes such as Tnfα and Ccl2 (Figure 7D), and genes related to fibrosis such as Acta2

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**Figure 2.** (See previous page). Western diet feeding reduces intestinal α1-2-fucosylation in mice. WT C57BL/6 mice were fed with either control diet and regular water (control diet groups) or Western diet combined with glucose (18.9 g/L) and fructose (23.1 g/L) in drinking water (Western diet groups) for 20 weeks. (A) Expression of Fut2 mRNA in ileum and colon tissue. (B) Expression of Fut4 mRNA in ileum and colon tissue. (C) Expression of Fut8 mRNA in ileum and colon tissue. (D) Representative images of colon tissues with immunohistochemistry staining for α1-2–linked (with Ulex Europaeus Agglutinin I), α1-3–linked (with Aleuria Aurantia Lectin), and α1-6–linked (with Aleuria Aurantia Lectin) fucosylation. WT mice were assigned to the 2'-FL–treated group and control group, and fed with either a control diet or a Western diet. In the 2'-FL–treated group, 2'-FL (2 g/L) was supplemented continuously in drinking water. The experimental diet and 2'-FL treatment lasted for 20 weeks. (E) Body weight, liver weight, plasma ALT levels, and representative images of H&E-stained liver tissue. Scale bar: 200 μm. Gene expression data are relative to ileum of control diet mice and all the data are presented as means ± SEM. **P < .001, ***P < .001, ****P < .0001. (A–O) The Student unpaired t test was used. (E) One-way analysis of variance followed by the Tukey post hoc test was used for comparison between groups. (A–D) Experiments performed in n = 11 on a control diet and n = 10 on a Western diet from 2–3 experiments, and for (E) experiments performed in n = 5–6 on a control diet and n = 10–14 on a Western diet from 2–3 experiments.
and Tgfβ1 (Figure 7E) were lower in Fut2−/− mice compared with WT mice following a Western diet for 20 weeks. Sirius red staining further showed the protective effect of Fut2 deficiency against Western diet–induced liver fibrosis (Figure 7F). Calorie-restricted and Western diet–fed Fut2−/− mice were fully protected from steatohepatitis as indicated by similar levels of ALT, steatosis, inflammation, and fibrosis parameters compared with control diet–fed groups (Figure 7). These findings indicate that Fut2 deficiency attenuates Western diet–induced steatohepatitis.
Protection From Obesity and Steatohepatitis Associated With Fut2 Deficiency Is Transmissible via Microbiota Exchange and Reduced by Antibiotic Treatment

Because intestinal α1-2-fucosylation is important for regulating the intestinal microbiota,16,18 we performed co-housing studies. Co-housing of mice in the same cage results in fecal microbiota transfer between mice. Strikingly, co-housing of WT littermates and Fut2-/− mice conferred protection from features of Western diet–induced obesity and steatohepatitis to WT mice (Figures 4A–E, 6A-B, 7A-F), indicating that the phenotype is transmissible via fecal microbiota transfer. Spots of α1-2-fucosylated glycans were

Figure 4. (See previous page). Fut2 deficiency protects mice from diet-induced obesity. Fut2−/− and WT littermates were fed with either a control diet or a Western diet for 20 weeks. Western diet–fed Fut2−/− mice had a significantly higher caloric intake than WT littermate mice. The total caloric intake of Fut2−/− mice was restricted to make it equal to the caloric intake of WT mice during Western diet feeding (calorie-restricted group). To facilitate fecal microbiota transfer between mice, freshly weaned WT and Fut2−/− mice were co-housed in the same cage and subjected to Western diet feeding. (A) Body weight and representative images for WT and Fut2−/− Western diet–fed mice. (B) Insulin tolerance test (ITT) was performed after 19 weeks of control or Western diet feeding. (C) Plasma cholesterol levels. (D) Plasma leptin levels. (E) Area under curve (AUC) of calorie intake over the course of the experiment. After 20 weeks of control or Western diet feeding, mice were housed in the comprehensive laboratory animal monitoring system metabolic cages for the measurement of metabolic data. (F) Energy expenditure was calculated by VO2 and respiratory exchange ratio (RER). (G) Rectal temperatures at room temperature. (H) Immunoblot for Ucp1 in brown adipose tissue. Data represent means ± SEM. *P < .05, **P < .01, ***P < .001, and ****P < .0001. One-way analysis of variance followed by the Tukey post hoc test was used for comparison between Western diet groups. Experiments were performed in n = 5–13 per group from 3–5 experiments. For the insulin tolerance test n = 13 in the WT Western diet group and n = 20 in the Fut2−/− Western diet group from 5 experiments. For the metabolic cages, n = 4–6 per group from 3 experiments.
observed in the intestine of Fut2<sup>−/−</sup> co-housed mice (likely owing to α1-2-fucosylated glycans from feces in coprophagic mice), but completely absent in control and Western diet–fed Fut2<sup>−/−</sup> mice. Co-housed and Western diet–fed WT mice showed lower expression of α1-2-fucosylated glycans compared with WT mice fed a control diet, but this was similar to Western diet–fed WT mice (Figure 3). Our co-housing studies indicate that the phenotype is transmissible via fecal microbiota transfer.

To further show a contribution of the intestinal microbiota, gut bacteria were reduced with antibiotics. WT and Fut2<sup>−/−</sup> mice on a Western diet for 12–13 weeks received antibiotics for an additional 5 weeks, while being continued on a Western diet. Antibiotic-treated WT and Fut2<sup>−/−</sup> Western diet–fed mice were no longer protected from obesity and steatohepatitis compared with vehicle-treated Fut2<sup>−/−</sup> mice because they gained more body weight and had more severe liver disease (Figure 8). Ucp1 protein expression in brown adipose tissue did not change in antibiotic-treated Fut2<sup>−/−</sup> mice compared with vehicle-treated Fut2<sup>−/−</sup> mice following Western diet feeding (data not shown). On the contrary, WT Western diet–fed mice treated with antibiotics lost body weight and showed improved steatohepatitis compared with vehicle-treated WT mice (Figure 8), which could be the result of a decrease in systemic lipopolysaccharide levels after antibiotic treatment as reported. We found a trend toward decreased Fut2 mRNA in the colon of Western diet–fed WT mice treated with antibiotics compared with vehicle-treated Western diet–fed WT mice (Figure 8D), which could contribute to lower body weight in antibiotic-treated WT mice. These findings further support an important role of the intestinal microbiota mediating the effect of Fut2 deficiency in protecting from diet-induced obesity and steatohepatitis.

**Fut2-Deficient Mice Show an Altered Plasma Metabolome and Intestinal Microbiome**

One possible mechanism for the protection from obesity and steatohepatitis of co-housed WT mice could be through transfer of beneficial intestinal metabolites and/or microbes associated with Fut2 deficiency. We therefore combined plasma metabolomics with fecal metagenomics. WT and Fut2<sup>−/−</sup> Western diet–fed mice showed different plasma metabolomic profiles (Figure 9A). One of the most prominent changes in plasma metabolites was found with bile acids. Total plasma bile acids not only were decreased significantly in Western diet–fed Fut2<sup>−/−</sup> mice compared with Western diet–fed WT mice (Figure 10A), but Fut2<sup>−/−</sup> mice had higher proportions of secondary and lower proportions of primary bile acids in plasma and the large intestine (cecum) than WT mice after feeding a Western diet (Figure 10B and C). The majority of bile acids were primary bile acids, and the proportions between primary and secondary bile acids were not different in the proximal and mid-small intestine (duodenum and jejunum) between WT and Fut2<sup>−/−</sup> Western diet–fed mice (Figure 11A), which indicates an important role of bile acid–metabolizing bacteria in the distal small and large intestine. Co-housed WT mice also showed a trend toward a higher proportion of secondary and lower proportion of primary bile acids in the cecum and plasma (Figure 10B and C). At baseline, WT and Fut2<sup>−/−</sup> mice not only had similar levels of plasma bile acids (Figure 10A), but also a similar composition of plasma and cecum bile acids (Figure 11B and C).

To evaluate the taxonomic composition and microbial diversity, shotgun metagenomic libraries of mouse fecal samples were sequenced and co-assembled to generate nearly complete genomes. Taxonomic analysis showed similar changes between WT and Fut2<sup>−/−</sup> mice in bacterial composition (Figure 9B) and diversity after Western diet feeding (Figure 9C). Functional analysis using mouse catalog genes showed that the relative abundance of bacterial enzyme 7α-hydroxysteroid dehydrogenases (7α-HSDH, encoded by the *hsdh* gene) participated in the conversion of primary into secondary bile acids, and was increased significantly in Western diet–fed Fut2<sup>−/−</sup> mice compared with Western diet–fed WT mice (Figure 10D). The relative abundance of the *hsdh* gene showed the same trend of increase in co-housed WT and Fut2<sup>−/−</sup> groups compared with WT mice on a Western diet (Figure 10D).

Taken together, Fut2<sup>−/−</sup> mice had a higher relative abundance of the bacterial enzyme 7α-HSDH, which is an important and broadly distributed enzyme in converting primary into secondary bile acids, and this might explain the different patterns of plasma and intestinal bile acids after Western diet feeding.

**Fut2 Deficiency Attenuates Western Diet–Induced Dysregulation of Bile Acid Metabolism**

To further study the impact of Fut2<sup>−/−</sup> deficiency on bile acid metabolism, we measured bile acids in different compartments. Consistent with lower plasma bile acids, Western diet–fed Fut2<sup>−/−</sup> mice showed lower total bile acids in the liver and a decreased bile acid pool size compared with Western diet–fed WT mice (Figure 12A). A decrease in the bile acid pool can be caused by reduced bile acid synthesis and/or by an increase in bile acid excretion. Indeed, we found that feces of Fut2<sup>−/−</sup> mice contained more bile acids than feces of WT mice after a Western diet (Figure 12B). *Scl10a2* (also called apical Na<sup>+</sup>-dependent bile salt transporter), which is responsible for the uptake of primary bile acids in the terminal ileum, was expressed similarly in all mouse groups (Figure 12C). These results indicate that an increased intestinal conversion of primary into secondary bile acids with a subsequent lower reuptake of secondary bile acids likely is responsible for increased fecal bile acid excretion.

In addition, Fut2<sup>−/−</sup> mice showed a decrease of cytochrome P450, family 7, subfamily a, polypeptide 1 (*Cyp7a1*) protein (Figure 12F), but increased cholesterol in the liver (Figure 12D) compared with WT mice after a Western diet, indicating that bile acid synthesis from cholesterol is lower in Fut2<sup>−/−</sup> mice. Hepatic cytochrome P450, family 8, subfamily b, polypeptide 1 (*Cyp8b1*) mRNA expression was not significantly different between mouse groups (Figure 12E).
Figure 6. Western diet–fed Fut2−/− mice have increased energy expenditure. Fut2−/− and WT littermates (normal groups and co-housed groups) were fed with either a control diet or a Western diet for 20 weeks. After 20 weeks of feeding mice were housed in the comprehensive laboratory animal monitoring system metabolic cages for the measurement of metabolic data, including VO₂, VCO₂, respiratory exchange ratio, rate of energy expenditure calculated by VO₂ and respiratory exchange ratio, and cumulative ambulatory counts for horizontal and vertical activity. (A) Metabolic parameters in dark cycles. (B) Metabolic parameters in light cycles. Data represent means ± SEM. *P < .05. One-way analysis of variance followed by the 2-stage step-up method of Benjamini, Krieger, and Yekutieli test was used for comparison between Western diet groups. Experiments were performed in n = 4–6 per group from 3 experiments.
Farnesoid X receptor (FXR, encoded by the \textit{Nr1h4} gene)–induced expression of fibroblast growth factor (Fgf)15 in the terminal ileum is known to suppress Cyp7a1 in the liver. Expression of intestinal \textit{Nr1h4} and \textit{Fgf15} mRNA was up-regulated in all Western diet–fed mice, but Western diet–fed WT mice had the highest levels (Figure 12G). Despite increased \textit{Fgf15}, Western diet–fed WT mice had the highest Cyp7a1 protein levels (Figure 12F), indicating that the negative feedback regulation of bile acid synthesis is nonfunctional. Cyp7a1 is regulated additionally by hepatic...
FXR. We therefore measured systemic FXR activity using a reporter assay. FXR activity was significantly higher in Western diet-fed WT mice than in Fut2+/− mice and control diet mice (Figure 12H). Changes that we have observed in Fut2+/− mice were similar in calorie-restricted Fut2+/− mice and co-housing groups, confirming the transmissibility of the phenotype (Figure 12A–G). These findings indicate that despite increased total bile acids, WT mice are not able to down-regulate bile acid synthesis and appear to be resistant to increased Fgfl5 and higher systemic FXR activity. In contrast, changes in intestinal bile acid metabolism associated with Fut2 deficiency results in increased fecal bile acid secretion, decreased bile acid synthesis, and a lower bile acid pool.

Supplementation of Exogenous α1-2-Fucosylated Glycans Exacerbates Steatohepatitis

To test whether we can overcome genetic Fut2 deficiency by dietary supplementation of α1-2-fucosylated glycans, Fut2+/− mice were administered 2′-FL together with a Western diet or control diet. Fut2+/− mice supplemented with 2′-FL gained significantly more body and liver weight (Figure 13A and B), and had a similar caloric intake as Fut2+/− mice fed a Western diet alone (Figure 14A). Western diet–fed Fut2+/− mice supplemented with 2′-FL showed increased liver injury (Figure 13C), higher plasma bile acids, a higher proportion of plasma primary bile acids and a lower proportion of plasma secondary bile acids (Figure 14D), increased hepatic steatosis (Figure 13D), and a higher expression of inflammatory and fibrosis-related genes, including Tnfα, Ccl2, and Col1a1 (Figure 14C) compared with Fut2+/− mice not receiving 2′-FL. Supplementation with 2′-FL caused a significant decrease in the proportion of deoxycholic acid (DCA) and lithocholic acid in plasma (Figure 14D), which are secondary bile acids generated by the enzyme 7α-HSDH,29–31 supportive of lower enzyme activity of 7α-HSDH under this condition. Consistent with results in chow diet–fed WT mice (Figure 2E), 2′-FL supplementation did not increase features of the metabolic syndrome including body weight gain and steatohepatitis in chow-fed Fut2−/− mice (Figure 13).

To determine whether L-fucose itself exerts any metabolic effects, WT mice were administered L-fucose together with the Western diet. Consistent with a previous report,52 Western diet–fed WT mice supplemented with L-fucose gained less body weight (Figure 15A). Western diet–fed WT mice supplemented with or without L-fucose had similar caloric intake (Figure 15B). Western diet–fed WT mice supplemented with L-fucose showed lower ALT levels (Figure 15C), lower liver weight (Figure 15D), and reduced hepatic steatosis as evidenced by hepatic triglycerides and H&E staining (Figure 15E). These findings indicate that α1-2-linked fucose but not L-fucose alone is responsible for the obesity and steatohepatitis-promoting effect.

Discussion

Epithelial α1-2-fucosylation is induced once commensal bacteria colonize the gut and is observed predominantly in the ileum and colon.14 In this study, we observed decreased intestinal α1-2-fucosylation in Western diet–fed mice, and restoration of intestinal α1-2-fucosylation with orally administered α1-2-fucosylated glycans exacerbated obesity and steatohepatitis. On the contrary, Fut2 deficiency attenuated diet-induced obesity and steatohepatitis despite a higher caloric intake than WT mice. Remarkably, protection from this phenotype (rather than the disease) is transmissible via fecal microbiota transfer and depletion of the gut microbiota by antibiotic treatment reduced differences between Western diet–fed WT and Fut2-deficient mice. Oral supplementation of α1-2-fucosylated glycans in the form of 2′-FL offsets the protective effect of Fut2 deficiency against features of the metabolic syndrome. We have linked Fut2 deficiency with changes in the microbial metabolism of bile acids. These data suggest a critical role of intestinal α1-2-fucosylation for the pathogenesis of obesity and steatohepatitis.

Fut2 polymorphism is associated with various disease conditions in human beings. Approximately 20% of Caucasians have nonfunctional variants of Fut2 on both alleles (also called nonsecretor phenotype), which is caused mainly by the single-nucleotide polymorphism rs601338.13 Secretor individuals have functional Fut2 alleles (genotype GG).34 Therefore, secretors can produce α1-2-fucosylated components, while nonsecretors lack this activity. Nonsecretor status increases susceptibility to primary sclerosing cholangitis (PSC) and Crohn’s disease.35,36 Lack of intestinal fucosylation results in altered intestinal microbiota, gut barrier function, and pathogen adhesion under disease conditions.18,37–42 For instance, in a chemical-induced colitis mouse model, Fut2-mediated intestinal α1-2-fucosylation protects against intestinal pathobionts such as Enterococcus faecalis and Citrobacter rodentium infection.18 In our
study, Fut2−/− mice (mimicking the nonsecretor status) were no longer protected from Western diet–induced obesity and steatohepatitis when α1,2-fucosylated glycans were restored in the intestine after oral administration of 2'-FL. Dietary supplementation of α1,2-fucosylated glycans (mimics transgenic overexpression of Fut2) not only abrogates the protective effects of genetic Fut2 deficiency, but also exacerbates the disease in Western diet–fed WT mice. Based on these findings, the down-regulation of intestinal α1,2-fucosylation in WT mice might be a defense mechanism against the harmful effects of a Western diet, and likely is regulated by changes in gut bacteria and the host’s response to a Western diet. Although 2'-FL is only known to be present in human milk, 2'-FL currently is used as a supplement to infant formula with claims to benefit infant growth. However, long-term effects of early life exposure to 2'-FL, especially in nonsecretor infants, remain unknown. Large patient population studies are needed to clarify the impact of Fut2 polymorphism on human obesity and NASH pathogenesis. Simple genome-wide association studies might be confounded by a functional down-regulation in Fut2 activity after eating a Western diet.

The limitation of our study was the use of a whole-body Fut2 knockout mouse, which cannot exclude the effect of Fut2 deficiency in organs other than the intestine. In addition to the distal small intestine, Fut2 is highly expressed in gallbladder epithelial cells, but not in liver bile duct cells. A possible effect of Fut2 deficiency in gallbladder epithelial cells on bile acid metabolism should be considered. In Fut2 nonsecretor individuals the absence of α1,2-fucosylated glycans was associated with higher disease progression and increased risk of biliary Candida infections in patients with PSC.43 Human hydrophobic bile acids induce more hepatobiliary damage in Fut2 knockout mice than WT mice.44 However, the role of Fut2 deficiency in all of these studies was associated with more biliary and liver disease, which is the opposite we found in our diet-induced obesity and steatohepatitis model. It is possible that the potential disadvantages of Fut2 deficiency for the hepatobiliary system is compensated by beneficial microbiota-mediated effects such as modulation of bile acids. To some extent, the whole-gene knockout mouse is closer to the physiological situation of a human nonsecretor status, but future studies with a tissue-specific deletion of Fut2 in intestinal epithelial cells are required.

Alterations of intestinal microbiota are involved in the pathogenesis of obesity and NASH.45,46 Bile acids are modified by the intestinal microbiota and act on both hepatic and extrahepatic tissues to maintain energy homeostasis through regulation of lipid and carbohydrate metabolic pathways.47 Thus, bile acids are the most promising signaling molecules that link obesity and NASH to intestinal microbiota. Increased serum bile acids are observed in patients with NASH, and excessive accumulation of bile acids in the liver induces hepatocyte death, inflammation, and progressive liver damage.48,49 Although 1 study reported that half of Fut2−/− mice had 40 times higher serum bile acids levels compared with WT mice,44 this was not identified in our study. Fut2−/− mice have similar plasma bile acids levels and bile acid components compared with WT littermate mice at baseline. After Western diet feeding, mice had increased liver cholesterol and this enhances the synthesis of bile acids by up-regulation of Cyp7a1. Biliary secretion of bile acids into the intestine and its reabsorption will be increased, resulting in an enlargement of the bile acid pool size. Provided that the negative feedback mechanism through intestinal FXR/Fgf15 is functioning properly—as we observed in our Western diet–fed Fut2−/− mice—increased intestinal bile acids will activate intestinal FXR, suppress Cyp7a1, and eventually decrease bile acid synthesis. In addition to this mechanism to reduce the bile acid pool, Western diet–fed Fut2−/− mice had increased fecal excretion of bile acids, likely owing to compositional changes and a higher proportion of secondary bile acids in the intestine. Functional metagenomic analysis showed a higher abundance of the bacterial gene encoding the enzyme 7α-HSDH in Western diet–fed Fut2−/− mice. 7α-HSDH is widely distributed in intestinal bacteria, including but not limited to Bacteroides, Clostridium, Escherichia coli, and Ruminooccus species, and participates in the oxidation and dehydroxylation of bile acids.44,45,48 Therefore, changes in primary and secondary bile acids in WT and Fut2−/− Western diet–fed mice might not be owing to a single bacterium, but rather caused by a bacterial community. Reduction of the bacterial hsdh gene has been reported in type 2 diabetes mellitus patients.50 Unlike Western diet–fed Fut2−/− mice, NASH patients have increased primary (mainly cholic acid and Chenodeoxycholic acid) and decreased secondary (mainly deoxycholic acid and lithocholic acid) plasma bile acids; a higher ratio of total secondary bile acid to primary bile acid decreases the likelihood of significant fibrosis.51 NASH and NAFLD patients also have an increased primary to secondary ratio of fecal bile acids.49 In summary, changes in the intestinal microbiota associated with Western diet–fed Fut2−/− mice

**Figure 8. (See previous page).** Protection from obesity and steatohepatitis associated with Fut2 deficiency is reduced by antibiotic treatment. WT and Fut2−/− mice fed a Western diet for 12–13 weeks were gavaged with antibiotics for 5 weeks to reduce gut microbiota. Control vehicle mice were gavaged with the same amount of sterile water. (A) Body weight changes and area under the curve of body weight increase during the course of the antibiotic treatment. (B) Total fecal DNA amount. (C) Plasma ALT levels. (D) Colon Fut2 mRNA level in WT mice. (E) Liver weight, hepatic triglyceride levels, and representative images of H&E-stained liver tissue. (F) Hepatic Tnfa, CcL2, and Col1a1 mRNA levels. Data represent means ± SEM. *P < .05, **P < .01, and ***P < .001. (A) *P < .05 compared with Fut2−/− Western diet group; **P < .05 compared with WT Western diet antibiotic group. One-way analysis of variance followed by the 2-stage step-up method of Benjamini, Krieger, and Yekutiel test was used. Scale bar: 200 μm. Experiments were performed in n = 5 per group from 2 experiments. AB, antibiotic treatment.
Figure 9. *Fut2* deficiency mice have altered plasma metabolome and intestinal microbiota. *Fut2*−/− and WT littermates were fed with either a control diet or a Western diet for 20 weeks. (A) There were 1984 different plasma metabolites quantified by untargeted metabolomics, and principal component analysis and hierarchical clustering of metabolomics data were performed using MetaboAnalyst 4.0. (B, C) Genomic DNA from mouse feces was extracted and purified for shotgun metagenomic sequencing. Rarefied reads from 73 samples were combined and assembled to generate near-complete genomes. The genomes were used to evaluate taxonomy and microbial diversity among the 7 groups. (B) Relative abundance of intestinal bacteria at genus level. (C) The β diversity of intestinal microbiota was analyzed by principal coordinate analysis. Experiments were performed in n = 10–13 per group from 3 experiments. PC, principal coordinate.
result in a decreased bile acid pool size by activating intestinal FXR signaling pathways and increasing fecal excretion of bile acids. This will prevent excessive accumulation of bile acids and liver damage.

The negative feedback mechanism through FXR/Fgf15 inhibits transcription of Cyp7a1 in hepatocytes and limits de novo synthesis of bile acids. Western diet–fed WT mice had a higher level of taurocholic acid (TCA) in the plasma (data not shown), which can act as an agonist of FXR and might contribute to increased serum FXR activity in these mice. However, the activation of FXR failed to inhibit bile acid synthesis in our Western diet–fed WT mice. Several studies have reported similar findings of increased FXR activation and increased bile acid synthesis in NAFLD/NASH patients and animal models. For instance, after an oral fat challenge, NAFLD patients without insulin resistance had an increase of plasma Fgf19 accompanied by a lower plasma level of the de novo bile acid synthesis marker C4 (7α-hydroxy-4-cholesten-3-one), while NAFLD patients with insulin resistance who had increased plasma Fgf19, a decrease of C4 was not observed. The most likely mechanism is that NAFLD/NASH patients had an impaired hepatic response to Fgf19 leading to dysregulation of bile acid synthesis and an increased total bile acid pool.53–56 GW4064 is a classic FXR agonist and was shown to be effective in repressing bile acid synthesis and hepatic steatosis in several previous studies. However, in our WT Western diet mice, GW4064 treatment failed to decrease bile synthesis and was not able to reduce obesity and hepatic steatosis (data not shown), this further supports that these mice were resistant to FXR/Fgf15 activation. The mechanism that causes nonresponse of Cyp7a1 to FXR activation is poorly understood. Increased plasma free fatty acids and hepatic microRNA-34a can prevent FXR-mediated repression of Cyp7a1 expression, however, further studies are needed to elucidate the mechanism of this resistance.

Western diet–fed Fut2+/− mice had a higher energy expenditure and an increase of nonshivering thermogenesis in brown adipose tissue indicated by a higher core body temperature and UCP-1 protein expression in brown adipose tissue than WT mice, and this is likely the main reason for the protection from diet-induced obesity and hepatic steatosis as increased caloric intake. One of the most significant changes found in Western diet–fed Fut2+/− mice is bile acid metabolism. Plasma bile acids, including both primary and secondary bile acids, had been shown to induce energy expenditure in brown adipose tissue. The most well-studied molecular mechanism that mediates the effects of bile acids on thermogenesis is G-protein–coupled bile acid receptor 1 (also known as takeda-G-protein-receptor-5 [TGR5]). However, we could not detect differences in TGR5 or differences of gastrointestinal hormones associated with appetite and energy expenditures, including glucagon-like peptide-1, peptide YY, and neuropeptide Y between Western diet–fed WT and Fut2+/− mice (data not shown). The observed increase in body temperature and activities likely was caused by an unknown mechanism, such as an altered set point of body temperature and emotional or behavioral changes, rather than by changes in bile acid metabolism. Further studies are needed to investigate the underlying mechanisms possibly linking other mechanisms to increased energy expenditure and thermogenesis in Fut2-deficient mice.

In summary, Fut2 deficiency protects mice from diet-induced features of the metabolic syndrome through increased energy expenditure, thermogenesis, and modified microbial metabolism of bile acids. α-1-2-fucosylation is an important host-derived regulator of intestinal microbiota and Fut2 plays a critical role for the pathogenesis of obesity and steatohepatitis in mice.

Materials and Methods

All authors had access to the study data and reviewed and approved the final manuscript.

Animal Models

Fut2+/− mice on a C57BL/6 background have been described (kindly provided by Dr Justin Sonnenburg, Stanford University). Heterozygous mice were used for breeding, and WT and Fut2+/− littermate mice were used in most experiments. WT C57BL/6 mice were purchased from Charles River (Wilmington, MA) and used in some experiments (Figures 1 and 15). Age-matched 8- to 9-week-old male WT and Fut2+/− mice were started on a Western diet (AIN-76A, TestDiet, St. Louis, MO, with a fat content of 20% and energy from fat was 40%), together with glucose (18.9 g/L) and fructose (23.1 g/L) in the drinking water for 20 weeks as described. In Figure 1, Western diet feeding was started at age 6–7 weeks. Control mice received standard chow diet. Mice had free access to food and water. Calorie-restricted Fut2+/− mice were provided the same amount of calories daily as their WT littermates on a Western diet. As previously described, WT and Fut2+/− mice fed a Western diet for 12–13 weeks were gavaged with vancomycin (4 mg/kg body weight [BW]), ampicillin (8 mg/kg BW), neomycin (8 mg/kg BW), metronidazole (8 mg/kg BW), and gentamicin (8 mg/kg BW) for 5 weeks. Control vehicle mice were gavaged with the same amount of water. The gavage was performed twice daily 5 d/wk and once daily 2 d/wk. All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). Supplementation of 2′-FL (Jennewein Biotechnologie GmbH, Rheinbreitbach, Germany) or L-fucose (GOLDBIO, St. Louis, MO) was performed by adding either of them in the drinking water at a final concentration of 2 g/L.

For all in vivo experiments, tissues were harvested from nonfasted mice with the exception of an insulin tolerance test for which mice werefasted for 7 hours, and the bile acid pool and FXR activity study, for which mice werefasted for 4–5 hours.

Insulin Tolerance Test

For the insulin tolerance test, mice were given an intraperitoneal injection of 1 U/kg insulin after 19 weeks on
a Western diet; blood glucose concentrations were measured after 7 hours of fasting before insulin injection, as well as 15, 30, 60, 90, and 120 minutes after injection.

**Metabolic Caging**

One week before being sacrificed, mice were housed in the Comprehensive Lab Animal Monitoring System.
Fut2−/− mice have altered plasma metabolome and intestinal microbiota. Fut2−/− and WT littermates were fed with either a control diet or a Western diet for 20 weeks. (A) Proportion of primary and secondary bile acids in small (duodenum and jejunum) and large intestine (cecum) in Western diet–fed mice. (B) Plasma bile acid composition in control diet–fed mice. (C) Cecum bile acid composition in control diet–fed mice. Experiments were performed in n = 10–13 per group from 3–5 experiments. The Student unpaired t test was used. Data represent means ± SEM. *P < .05.

Figure 11. Bile acid composition. Fut2−/− and WT littermates were fed with either a control diet or a Western diet for 20 weeks. (A) Proportion of primary and secondary bile acids in small (duodenum and jejunum) and large intestine (cecum) in Western diet–fed mice. (B) Plasma bile acid composition in control diet–fed mice. (C) Cecum bile acid composition in control diet–fed mice. Experiments were performed in n = 10–13 per group from 3–5 experiments. The Student unpaired t test was used. Data represent means ± SEM. *P < .05.
(Columbus Instruments, Columbus, OH) for approximately 60 hours to measure VO₂ (mL/kg/min), VCO₂ (mL/kg/min), respiratory exchange ratio (VCO₂/VO₂), heat production (kcal/kg/min; calculated as VO₂ × \[3.815 + (1.232 \times \text{respiratory exchange ratio})\]) and activity (infrared beam breaks in the x and y planes) as previously described. All mice had free access to food and water while in metabolic cages.
Biochemical Assays

Levels of plasma ALT were measured using the Infinity ALT kit (Thermo Scientific, Waltham, MA). Plasma and hepatic triglyceride and cholesterol levels were measured using the Triglyceride Liquid Reagents Kit (Pointe Scientific, Canton, MI) and Cholesterol Liquid Reagents kit (Pointe Scientific), respectively. Plasma levels of leptin were measured using the mouse leptin enzyme-linked immunosorbent assay kit according to the manufacturer’s protocol (Crystal Chem, Elk Grove Village, IL). Total bile acids and the bile acid pool were quantified using a Mouse Total Bile Acid Kit (Crystal Chem) as described. For the total bile acid pool, total liver bile acids, total gallbladder bile acids, and total bile acids from the small intestine and cecum, contents were measured and calculated per gram of body weight.

Staining Procedures

Formalin-fixed and paraffin-embedded livers were stained with H&E (Leica Biosystems, Inc, Buffalo Grove, IL) or 0.1% picrosirius red (Sigma-Aldrich) using standard staining protocols. Sirius red–positive area was quantitated by image analysis software ImageJ (National Institutes of Health, Bethesda, MD). The colon sections were treated with 0.1% H$_2$O$_2$ (Sigma-Aldrich) for 30 minutes and blocked with avidin and biotin (Vector, Torrance, CA) for 15 minutes each. After blocking with 1% bovine serum albumin for 5 minutes, colon sections were incubated with biotinylated Ulex Europaeus Agglutinin I (Vector) or biotinylated Aleuria Aurantia Lectin (Vector) overnight at 4°C. Sections then were incubated with streptavidin, horseradish peroxidase for 30 minutes, and followed by 3, 3’-diaminobenzidine solution (Vector) for 2 minutes and hematoxylin for 1 minute.

Real-Time Reverse-Transcription Quantitative PCR

RNA was extracted from mouse tissues, and complementary DNA was generated as described. Quantitative PCR was performed with iTaq universal SYBR Green Supermix (Bio-Rad, Hercules, CA) using a StepOnePlus thermocycler real-time PCR system. Primer sequences for mouse genes were obtained from the National Institutes of Health qPrimerDepot and are listed in Table 1. The values of mouse gene expression were normalized to 18S.

Immunoblotting

Liver or brown adipose tissue were homogenized in RIPA buffer, supplemented with protease inhibitor, and used for immunoblotting. Immunoblot analysis was performed using anti-Cyp7a1 (Abcam, Cambridge, MA), anti-Ucp1 (Abcam, CA) antibodies. Densitometry of immunoblot analysis was performed using ImageJ software (National Institutes of Health).

Cell Luciferase Assay

For luciferase assay, FXRE-Luc plasmids (FXR responsive element) were transfected into CV1 cells. Plasma was diluted with culture medium with a final concentration of 10% in volume and added to the cell culture. Luciferase activities were measured using a dual-luciferase reporter kit (Promega, Madison, WI) as previously reported.

Shotgun Metagenomic Sequencing

DNA sequencing. Total genomic DNA from mouse feces was extracted using the DNeasy PowerSoil Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions as described. Purified DNA was prepared for shotgun metagenomic sequencing using the Nextera XT library (Illumina, San Diego, CA) preparation method with 2 rounds of 0.7× ratio bead-based size selection on an Apollo 324 liquid handler (Takara Bio USA, Mountain View, CA) to generate an average fragment size of 800 base pairs (bp). Libraries were quality-assessed using quantitative PCR and a Bioanalyzer (Agilent Technologies, Santa Clara, CA), and subsequently sequenced on a NovaSeq 6000 S2 flow cell using a 300 cycle (2 × 150 bp) kit, loading 400 pmol/L of pooled library with 1% spike-in of φX174 DNA. The target sequencing depth was 5 Gbp (giga-base pair) per sample.

Data analysis. An average of 29.6 million reads were generated per library. Adapters were trimmed from the Illumina data using Trimmomatic v0.36. Samples were filtered of possible mouse contamination by aligning the trimmed reads against reference databases using Bowtie2 v2.2.3 with the following parameters (-D 20 -R 3 -N 1 -L 20 –very-sensitive-local).

For functional analysis, we used a previously constructed mouse gut microbiome database, comprising approximately 2.6 million nonredundant genes. Non-mouse trimmed reads were aligned to the mouse catalog genes using Bowtie (~very-sensitive) with an average
Figure 13. Restoration of α1-2-fucosylation in the intestine exacerbates diet-induced steatohepatitis in Fut2-deficient mice. Fut2−/− mice were assigned to the 2'-FL–treated group and control group, and fed with either a Western diet or a control diet. In the 2'-FL–treated group, 2'-FL (2 g/L) was supplemented continuously in drinking water. The experimental diet and 2'-FL treatment lasted for 20 weeks. (A) Body weight increase during 20 weeks. (B) Body weight and liver weight. (C) Plasma ALT levels. (D) Hepatic triglycerides and H&E-stained liver tissue. Data represent means ± SEM. *P < .05, **P < .01, ***P < .001, and ****P < .0001. (A) The Student unpaired t test was used for comparison between 2 Western diet groups with or without 2'-FL feeding. (B–D) One-way analysis of variance followed by the Tukey post hoc test was used for comparison between different groups. Scale bar: 200 μm. Experiments were performed in n = 12–16 per group from 3 experiments in Western diet groups and in n = 4–5 per group from 2 experiments in control diet groups. AUC, area under the curve.
Figure 14. Restoration of α1-2-fucosylation in the intestine exacerbates diet-induced steatohepatitis in Fut2-deficient mice. Fut2−/− mice were assigned to the 2'-FL–treated group and the control group, and fed with either a Western diet or a control diet. In the 2'-FL–treated group, 2'-FL (2 g/L) was supplemented continuously in drinking water. The experimental diet and the 2'-FL treatment lasted for 20 weeks. (A) Calorie intake. (B) Plasma bile acid levels and proportion of primary and secondary bile acids in plasma. (C) Hepatic Tnfα, Ccl2, and Col1a1 mRNA levels. (D) Relative amount of DCA and lithocholic acid (LCA) in total plasma bile acids. Data represent means ± SEM. *P < .05, **P < .01, ***P < .001, and ****P < .0001. The Student unpaired t test was used. Experiments were performed in n = 5–16 per group from 2–3 experiments. AUC, area under the curve.
Figure 15. Fucose feeding in WT Western diet-fed mice attenuates body weight increase. WT mice were assigned to L-fucose supplementation or control group, and fed with a Western diet for 20 weeks. In the L-fucose-treated group, L-fucose (2 g/L) was added continuously into the drinking water. (A) Body weight. (B) Area under curve (AUC) of calorie intake over the course of the experiment. (C) Plasma ALT levels. (D) Liver weight. (E) Hepatic triglyceride levels and representative images of H&E-stained liver tissue. Data represent means ± SEM. *P < .05. The Student unpaired t test was used. Scale bar: 200 μm. Experiments were performed in n = 9 per group from 2 experiments.
reads. Merged and unmerged reads were assembled using the number of reads, the libraries were rare

evaluated using CheckM v1.0.7.67 Taxonomic classification (closest phylogenetic neighbor) was assessed using
(Kyoto Encyclopedia of Genes and Genomes database) was compared using the Kegg Onthology K02781 (carbo-

49x197] reads was compared using the Kegg Onthology K02781 (carbohydrate metabolism) as the reference frame.70 The choice of the reference frame was made using Songbird tool,71 with the following parameters (–formula “genotype_treatment”, –epochs 10000, –differential-prior 0.5, –summary-interval 1). Shotgun metagenomic data are available at the Sequence Read Archives BioProject PRJNA614498.

Table 1. Sequences of Quantitative PCR Primers

| Gene         | Primer | Sequence                        |
|--------------|--------|---------------------------------|
| Mouse 18S    | F      | 5′-AGTCCCTGGCCCTTGGTACACA-3′    |
|              | R      | 5′-CGATCCACGGGCGCTCAG-3′        |
| Mouse Fut2   | F      | 5′-GGTTGGATGATGGTTGGAAGTC-3′    |
|              | R      | 5′-TTCCCTGTATCACCAAGCAG-3′      |
| Mouse Fut4   | F      | 5′-AAATCCCTATTTCCCTGGT-3′       |
|              | R      | 5′-CCAGGGGAAAGGAGGTTAAG-3′      |
| Mouse Fut8   | F      | 5′-TGCTTGAAATCGTGCTTGA-3′       |
|              | R      | 5′-GGCGCTCGTAAGTTTCTGACT-3′     |
| Mouse Tnfα   | F      | 5′-AGGGTCTGGGCCATAGA-3′         |
|              | R      | 5′-CCACACGGCTTCTCGTCTAC-3′      |
| Mouse Ccl2   | F      | 5′-ATTGGGATCATCTGTGCTG-3′       |
|              | R      | 5′-CCTGCTGTCCACAGTGCC-3′        |
| Mouse Acta2  | F      | 5′-GTTGAGTGGTGCTCTGTCA-3′       |
|              | R      | 5′-ACGGGACGGACATGGAAG-3′        |
| Mouse Tgfb1  | F      | 5′-GGAGGCGGCCGTGATACAC-3′       |
|              | R      | 5′-CAAACCGAGTCCTCTCCTAAA-3′     |
| Mouse Nr1h4  | F      | 5′-GAAACTGAACATCGGGTTAT-3′      |
|              | R      | 5′-CGCCGCGAGAATTTCTAATAG-3′     |
| Mouse Fgf15  | F      | 5′-GAGGACCGAAAAAGAACGAATT-3′    |
|              | R      | 5′-ACGTCCCGTTGATGCACATC-3′      |
| Mouse Col1a  | F      | 5′-TAGGCCATTTGTGATGCGAC-3′      |
|              | R      | 5′-ACATGTTGGCAGTTTGCGACC-3′     |
| Mouse Slc10a2| F      | 5′-TGTTGTGACGAGAAGGACG-3′       |
|              | R      | 5′-GCTATTGGAATAGATTGCCG-3′      |
| Mouse Cyp8b1 | F      | 5′-CATGGCTTCCGGGGAAGAATA-3′     |
|              | R      | 5′-TCTTAATGATGGGCGGAAAG-3′      |

F, forward; R, reverse.

For de novo genome assembling, overlapped reads were merged using Flash version 1.2.11.64 Because of the massive number of reads, the libraries were rarefied to 4 million reads. Assembled and unmerged reads were assembled using Spades v3.12.065 with the following parameters (–k 25 meta–merge). Differential binning was performed using MetaBat2 v2.12.1,66 with minimum contig length of 1500 bp. Bin quality (completeness and contamination) was evaluated using CheckM v1.0.7.57 Taxonomic classification (closest phylogenetic neighbor) was assessed using RASTTk.68 In brief, RAST uses a set of unique protein sequences to assign the closest related neighbor. Genome annotations were performed using Prokka v1.1169 with default parameters.

**Microbiome statistical analysis.** Microbial diversity was estimated using R package vegan v2.5-2. Plots generated using R package ggplot2 v3.3.2. Differential relative abundance was compared through a log ratio of the counts through Qurro v0.7.1, with statistical significance evaluated through a nonparametric Wilcoxon rank sum test using R scripts. For comparisons of abundance, a log ratio of counts was compared using the Kegg Onthology K02781 (carbohydrate metabolism) as the reference frame.70 The choice of the reference frame was made using Songbird tool,71 with the following parameters (–formula “genotype_treatment”, –epochs 10000, –differential-prior 0.5, –summary-interval 1). Shotgun metagenomic data are available at the Sequence Read Archives BioProject PRJNA614498.

**Untargeted Metabolomics of Plasma Samples**

**Sample preparation.** Briefly, 90 µL of methanol/acetonitrile (3:1) solution (containing 0.6 µg/mL L2-chlorophenylalanine and 6.0 µg/mL ketoprofen as the internal standards) was added to 30 µL plasma and vortexed for 30 seconds. Then, at 4°C, the mixture was centrifuged for protein precipitation (13,000 rpm, 10 min). After that, duplicate supernatants (each for 45 µL) were transferred and dried under nitrogen at room temperature. One of the resulting residues was redissolved in 60 µL of 50% aqueous acetonitrile for untargeted analysis in positive ion mode, while the other was immediately stored at -80°C (for the negative ion mode). Quality control samples were obtained by pooling equal aliquots (10 µL) from each plasma sample and pretreated with the same procedure.

**Liquid chromatography-quadrupole-time-of-flight–mass spectrometry analysis.** Untargeted analyses were performed using an Agilent 1290 infinity liquid chromatography (LC) system coupled to an Agilent 6545 quadrupole-time-of-flight mass spectrometer (MS) equipped with an electrospray ionization (ESI) source operating in both positive and negative ion modes. Chromatographic separation was carried on an Acuity UPLC HSS T3 column (Waters, Wexford, Ireland) (2.1 × 100 mm, 1.8 µm) with a flow rate of 0.4 mL/min at 50°C. The mobile phase used for ESI+ consisted of 0.1% aqueous formic acid (mobile phase A) and acetonitrile (mobile phase B). For ESI-, the mobile phase consisted of (mobile phase A) 10 mmol/L ammonium acetate aqueous solution and (mobile phase B) 10 mmol/L ammonium acetate aqueous solution. The ionization voltage was 1.5 KV. MS parameters were set as follows: drying gas temperature, 320°C; drying gas flow rate, 8 L/min; nebulizer gas, 35 psi; fragmental voltage, 120 V; and capillary voltage, 3500 V. A full scan from 50 to 1050 m/z was acquired for each sample under the high-resolution mode (extended dynamic range, 2 GHz).

**Data analysis.** All the acquired spectra were first converted to mz data format and then the XCMS package (available from http://metlin.scripps.edu/download) of R program was run for data pretreatment including peak
Targeted Metabolomics of Plasma Samples

Plasma pretreatment. A 30-μL aliquot of plasma was mixed with 10 μL of internal standards working solution (9 μg/mL of tauro-β-muricholic acid (T-β-MCA)-d4, 0.9 μg/mL of ω-MCA-d5, 3.6 μg/mL of β-MCA-d5, 4.5 μg/mL of cholic acid (CA)-d4, 1.8 μg/mL of DCA-d4, 9 μg/mL of TCA-d4, and 45 ng/mL of glycocholic acid (GCA)-d4). Then, 80-μL aliquots of methanol solution were added and vortexed for 2 minutes to extract the bile acids. After centrifugation for 10 minutes at 13,000 rpm, 4°C, 100 μL of supernatant carefully was transferred and dried with continuous nitrogen. Finally, the residue was reconstituted in 60 μL of 50% aqueous acetonitrile solution (containing 0.1% formic acid) and 5 μL was injected for further LC-MS/MS analysis.

LC-MS/MS analysis. Targeted analyses were performed using an LC-20A system coupled to a triple quadrupole mass spectrometer (LC-MS/MS 8050; Shimadzu, Nakagyo Ward, Kyoto, Japan) operating in negative ion mode. The high-performance liquid chromatography (HPLC) separation was achieved on an Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm) maintained at 45°C. Pure water and water/acetonitrile (v/v = 1:9) both containing 1 mmol/L ammonium acetate were used as mobile phase A and B, respectively, at a flow rate of 0.4 mL/min. The gradient elution program was 5%-25% B at 0–1 minute, 25%-30% B at 1–9 minutes, 30%-40% B at 9–10 minutes, 40%-45% B at 10–17 minutes, 45%-95% B at 17–18.5 minutes, and 95% B held for 2 minutes, and then back to the initial conditions with 3 minutes for equilibration. The ESI source parameters were as follows: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; drying gas flow, 10 L/min; interface temperature, 300°C; DL temperature, 250°C; and heat block temperature, 400°C.

Targeted quantification. A total of 10 bile acids in plasma were measured quantitatively based on a stable isotope-labeled internal standard calibration strategy. Multiple reaction monitoring mode was selected, thus allowing more precise results and the detailed ion transitions monitored were as follows: T-β-MCA, m/z 514→80; T-β-MCA-d4, m/z 518→80; ω-MCA, m/z 407→407; ω-MCA-d5, m/z 412→412; β-MCA, m/z 407→407; β-MCA-d5, m/z 412→412; DCA, m/z 391→391; DCA-d4, m/z 395→395; CA, m/z 407→407; CA-d4, m/z 411→411; TCA, m/z 514→124; TCA-d4, m/z 518→124; GCA, m/z 464→74; GCA-d4, m/z 468→74; TUDCA, m/z 498→80; TDCA, m/z 498→80; and THDCA, m/z 498→80. Standard solutions over a wide concentration range of 800-fold were prepared for the linearity investigation. All the standard curves showed good linearity with regression coefficients r² > 0.99. The accuracy was assessed at high, moderate, and low concentration levels and calculated as recoveries ranged from 80% to 120%, indicating high accuracy of the LC-MS/MS approach. The interday precision (1 replicate of quality control sample analyzed on each of 3 days) and intraday precision (3 replicates analyzed on the same day) also were measured and calculated from the relative SD (RSD, % = SD of peak area ratio/mean of peak area ratio × 100). All the bile acid metabolites with intraday and interday precision (RSD) less than 8% and 15%, respectively, further confirmed the stability of the analytical platform. The lower limit of quantification was determined at a signal-to-noise ratio greater than 10.

Targeted Metabolomics of Cecum Content Samples

Sample preparation. Cecum contents were homogenized and dried in a vacuum centrifuge at 37°C until the weight was stable. Fifteen milligrams of homogenized intestinal content was extracted in 1.0 mL 75% ethanol for 1 hour. The extracts were centrifuged at 16,200 × g at 4°C for 10 minutes subsequently. Once the supernatants were prepared, an aliquot of a 900-μL sample of the supernatant was evaporated to dryness under reduced pressure at 45°C using the EZ2 Plus Solvent Evaporation Station (SP Scientific, Warminster, PA). The residue was dissolved by addition of 200 μL acetonitrile and the supernatant was injected into the HPLC-MS/MS system for analysis.

LC-MS/MS analysis. Targeted analyses were performed using an LC-20A system coupled to a triple quadrupole mass spectrometer (LC-MS/MS 8050; Shimadzu) operating in negative ion mode. The HPLC separation was achieved on an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) maintained at 45°C. Pure water and water/acetonitrile (v/v = 1:9) both containing 0.1% formic acid were used as mobile phase A and B, respectively, at a flow rate of 0.3 mL/min. The gradient elution program was 30%-65% B at 0–3 minutes, 65%-70% B at 3–10 minutes, 65%-70% B at 10–12 minutes, 70%-85% B at 12–13 minutes, and 85%-30% B at 13–14 minutes. The ESI source parameters were as follows: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; drying gas flow, 10 L/min; interface temperature, 300°C; DL temperature, 250°C; and heat block temperature, 400°C.

Targeted quantification. A total of 12 bile acids in plasma were measured quantitatively based on a stable isotope-labeled internal standard calibration strategy. The multiple reaction monitoring mode was selected, thus allowing more precise results and the detailed ion transitions monitored were as follows: T-β-MCA, m/z 514→80; T-β-MCA-d4, m/z 518→80; ω-MCA, m/z 407→407; ω-MCA-d5, m/z 412→412; β-MCA, m/z 407→407; β-MCA-d5, m/z 412→412; DCA, m/z 391→391; DCA-d4, m/z 395→395; CA, m/z 407→407; CA-d4, m/z 411→411; TCA, m/z 514→124; TCA-d4, m/z 518→124; GCA, m/z 464→74; GCA-d4, m/z 468→74; TUDCA, m/z 498→80; TDCA, m/z 498→80; and THDCA, m/z 498→80. Standard solutions over a wide concentration range of 800-fold were prepared for the linearity investigation. All the standard curves showed good linearity with regression coefficients r² > 0.99. The accuracy was assessed at high, moderate, and low concentration levels and calculated as recoveries ranged from 80% to 120%, indicating high accuracy of the LC-MS/MS approach. The interday precision (1 replicate of quality control sample analyzed on each of 3 days) and intraday precision (3 replicates analyzed on the same day) also were measured and calculated from the relative SD (RSD, % = SD of peak area ratio/mean of peak area ratio × 100). All the bile acid metabolites with intraday and interday precision (RSD) less than 8% and 15%, respectively, further confirmed the stability of the analytical platform. The lower limit of quantification was determined at a signal-to-noise ratio greater than 10.
solutions over a wide concentration range were prepared for the linearity investigation. All of the standard curves showed good linearity with regression coefficients $r^2 > 0.99$. The accuracy was assessed at high, moderate, and low concentration levels and calculated as recoveries ranged from 80% to 120%, indicating high accuracy of the LC-MS/MS approach. The interday precision (1 replicate of quality control sample analyzed on each of 3 days) and intraday precision (3 replicates analyzed on the same day) also were measured and calculated from the RSD (% intraday precision (3 replicates analyzed on the same day) and quality control sample analyzed on each of 3 days) and gave results ranging from 80% to 120%, indicating high accuracy of the analytic platform. The lower limit of quantification was determined at a signal-to-noise ratio > 10.

**Statistical Analysis**

All data were expressed as means ± SEM unless otherwise specified. For comparison of 2 groups, the Student unpaired t test was used. For comparisons of more than 2 groups, 1-way analysis of variance was used followed by the Tukey post hoc test. Analysis of metabolomic data was performed with R (V.3.5.1; 2018 the R Foundation for Statistical Computing), and principal component analysis and hierarchical clustering of metabolomics data were performed using MetaboAnalyst 4.0. All the other analyses were performed with GraphPad Prism V.7.0. (La Jolla, CA). A P value less than .05 was considered significant.

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