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Long-Term β-galacto-oligosaccharides Supplementation Decreases the Development of Obesity and Insulin Resistance in Mice Fed a Western-Type Diet

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Scope: The gut microbiota might critically modify metabolic disease development. Dietary fibers such as galacto-oligosaccharides (GOS) presumably stimulate bacteria beneficial for metabolic health. This study assesses the impact of GOS on obesity, glucose, and lipid metabolism.
Methods and results: Following Western-type diet feeding (C57BL/6 mice) with or without β-GOS (7% w/w, 15 weeks), body composition, glucose and insulin tolerance, lipid profiles, fat kinetics and microbiota composition are analyzed. GOS reduces body weight gain ($p < 0.01$), accumulation of epididymal ($p < 0.05$), perirenal ($p < 0.01$) fat, and insulin resistance ($p < 0.01$). GOS-fed mice have lower plasma cholesterol ($p < 0.05$), mainly within low-density lipoproteins, lower intestinal fat absorption ($p < 0.01$), more fecal neutral sterol excretion ($p < 0.05$) and higher intestinal GLP-1 expression ($p < 0.01$). Fecal bile acid excretion is lower ($p < 0.01$) in GOS-fed mice with significant compositional differences, namely decreased cholic, α-muricholic, and deoxycholic acid excretion, whereas hyodeoxycholic acid increased. Substantial changes in microbiota composition, conceivably beneficial for metabolic health, occurred upon GOS feeding.
Conclusion: GOS supplementation to a Western-type diet improves body weight gain, dyslipidemia, and insulin sensitivity, supporting a therapeutic potential of GOS for individuals at risk of developing metabolic syndrome.

1. Introduction

The world population is facing an epidemic of metabolic syndrome-related disease, largely due to a growing consumption of "Western" diets and a sedentary lifestyle.[1] Unhealthy nutrition induces obesity with an associated increase in oxidative stress, fat accumulation, inflammation, and insulin resistance among other metabolic dysregulations. Chronic non-communicable diseases such as type 2 diabetes, non-alcoholic fatty liver disease, and cardiovascular disease are serious adverse consequences of prolonged exposure to such conditions.[2,3] Accumulating observations indicate that changes in gut microbiota composition induced by Western-style diets play an important role in modifying the development of metabolic syndrome. Significant shifts in microbiota composition have been associated with inflammation, obesity, and metabolic dysregulation.[4]

Dietary fibers are a vital source of energy for gut microbial populations. Fibers have been shown to influence the composition of the gut microbiota and thereby

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the production of bioactive metabolites such as short-chain fatty acids (SCFA), secondary bile acids, vitamins, and more. These bioactive metabolites have been suggested to exert various metabolic effects on the host.[5]

Galacto-oligosaccharides (GOS) are dietary fibers derived from lactose using either α- or β-galactosidase enzymes.[6] GOS is a soluble fiber widely used for its potential to alter gut microbiota composition by stimulating growth of bacteria supposedly beneficial for metabolic health such as members of the genera *Bifidobacterium* and *Lactobacillus*. Different varieties of GOS have been utilized in a limited number of clinical studies. Under free living conditions it has been shown that GOS supplementation in healthy elderly as well as overweight volunteers can lead to altered gut microbiota composition and improvement of biomarkers of systemic inflammation,[7,8] while no improvements of glucose tolerance were detected either by clamp techniques in obese, prediabetic subjects receiving β-GOS[9] or by OGTT in healthy, young volunteers receiving α-GOS.[10] However, thus far, the long-term effects of GOS on the development of obesity and insulin resistance on the background of a Western-type high fat diet have neither been studied in humans nor in preclinical models. Therefore, the present work aimed to investigate long-term metabolic effects of β-GOS supplementation to a Western-type diet in vivo in mice, including an evaluation of potential underlying mechanisms.

2. Results

2.1. Dietary GOS Supplementation Reduced the Development of Body Weight Gain, Dyslipidemia, and Insulin Resistance

Prior to the dietary intervention both groups of animals were matched for age and body weight. The GOS-containing diet was tolerated well, mice did not experience loose stools or had any other visible abnormality; physical activity was not different from the control group (control vs GOS-fed mice during the day, 13.5 ± 6.5 vs $13.7. \pm 5.7$ m, $p = 0.97$; during the night, 25.8 ± 7.5 vs 22.0 ± 7.6 m, $p = 0.48$). A significantly lower body weight gain (between 3–12%, **Figure** 1A, $p < 0.01$) was observed from the second week onward, while food intake in both groups remained unchanged (Figure 1B). Using NMR analysis a lower fat mass was observed in GOS-fed mice compared to the control group, but the difference did not reach statistical significance (−17%, Figure 1C, $p = 0.055$). Upon sacrifice, weighing of individual fat depots demonstrated that GOS feeding lead to significantly lower epididymal (−12%, $p < 0.05$) and perirenal (−29%, $p < 0.01$) fat accumulation (Figure 1D). Glucose tolerance tests performed at the end of the dietary intervention indicated no differences between the groups (Figure 1E). Development of insulin resistance, however, was reduced in the GOS-supplemented groups (Figure 1F) with the area under the curve (AUC) being significantly lower in GOS-fed animals (−20%, Figure 1G, $p < 0.05$). Interestingly, GOS supplementation increased in the proximal intestine the *mRNA* expression of proglucagon, the gene encoding glucagon-like peptide-1 (Glp-1), an incretin hormone responsible for stimulating insulin secretion, which is subsequently generated by proteolytic processing (+66%, **Table 1**, $p < 0.001$). Correspondingly, circulating Glp-1 levels were higher in GOS-fed mice compared with controls (2.62 ± 0.25 vs 1.30 ± 0.15 ng L^{-1}, respectively, $p < 0.01$). Cecal levels of the short-chain fatty acids acetate (45.9 ± 5.3 vs 27.1 ± 2.5 µmol g^{-1}, respectively, $p < 0.05$) and butyrate (9.6 ± 1.3 vs 5.1 ± 0.7 µmol g^{-1}, respectively, $p < 0.05$) as well as levels of lactate (10.1 ± 1.3 vs 4.3 ± 1.2 µmol g^{-1}, respectively, $p < 0.01$) were higher in the GOS receiving group, while propionate levels showed no significant change (13.7 ± 2.0 vs 9.1 ± 1.4 µmol g^{-1}, respectively).

At the end of the dietary intervention, plasma total cholesterol was lower in the GOS-fed group (−20%, Figure 1H, $p < 0.05$). FPLC analysis of the plasma indicated that the reduction in total cholesterol was largely contributed by a reduction in low-density lipoprotein (LDL) particles in GOS-fed mice (Figure 1I). This change in plasma lipids occurred in the face of decreased LDL receptor *mRNA* expression in the liver of the GOS receiving mice (Table 1). Furthermore, plasma triglyceride levels were significantly lower in the GOS-fed group (−40%, Figure 1J, $p < 0.05$). At week 15, GOS-fed mice also showed a trend toward a lower liver/body weight ratio (Figure 1K, $p = 0.06$). In GOS-fed mice, hepatic triglyceride levels tended to be lower (−33%, IL, $p = 0.065$), whereas hepatic cholesterol levels remained unchanged (Figure 1M) compared to the control group.

2.2. GOS Supplementation Did Not Alter Energy Expenditure or the Respiratory Exchange Ratio

In order to investigate the cause of lower body weight gain in the face of unchanged food intake, we first analyzed brown adipose tissue (BAT) for potential indications for a change in its thermogenic capacity. Electron microscopy of BAT showed no substantial change in mitochondrial morphology and lipid droplets (**Figure 2**A). *mRNA* expression of several relevant genes remained unchanged (Figure 2B). However, we detected a significant increase in transcription of the gene encoding for uncoupling protein 1 (*Ucp1*), a mitochondrial carrier protein of BAT involved in heat generation by disruption of the proton gradient during respiration (Figure 2B, $p < 0.05$).

Because of the higher expression of *Ucp1* we next performed indirect calorimetry to investigate whether mice on GOS supplementation had an altered energy metabolism. We measured energy expenditure (EE) and calculated respiratory exchange ratios (RER) based on oxygen consumption and carbon dioxide production. Both groups had comparable RER during the light hours when the mice were resting as well as during the night hours when the mice were active (Figure 2C–E). Control and GOS-fed mice also showed similar EE during day and night hours (Figure 2F,G). Thus, the increase in *Ucp1 mRNA* expression in BAT did not translate into a physiologically meaningful increase in energy metabolism.

2.3. GOS Altered Fecal Neutral Sterol and Bile Acids Profiles

We investigated the effect of GOS on the fecal excretion of cholesterol and bile acids including their microbiota-derived products. At the end of the dietary intervention both groups had similar fecal mass output (**Figure 3**A). In the neutral sterol profile

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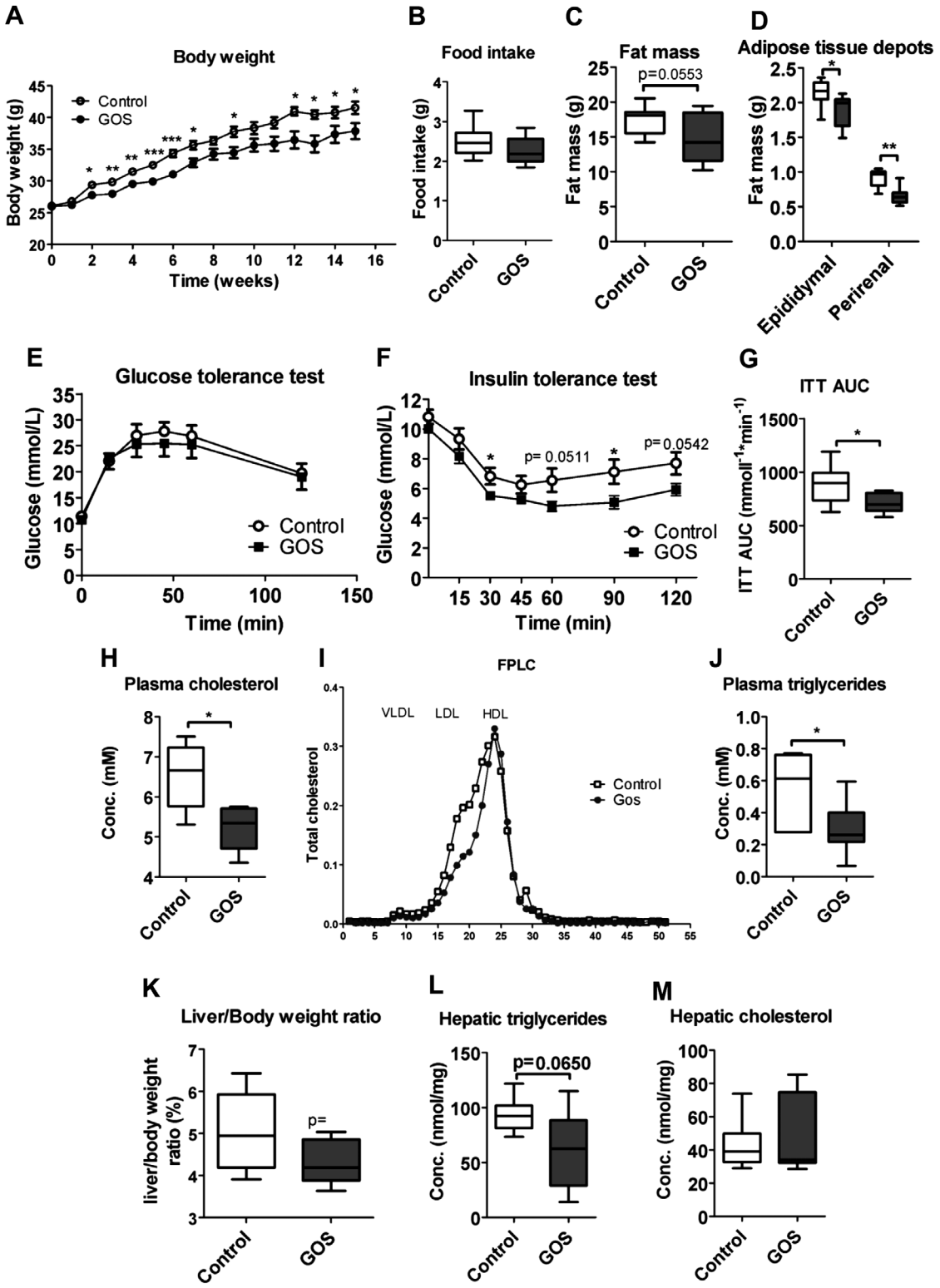

Figure 1. GOS supplementation reduces the development of metabolic syndrome related disease phenotypes. A) body weight gain; B) food intake at the end of the dietary intervention; C) fat mass; D) adipose fat depots; E) glucose tolerance test (GTT) performed at the end of the dietary intervention on 6 h-fasted mice; F) insulin tolerance test (ITT) performed at the end of the dietary intervention on 4 h-fasted mice; G) total glucose area under the curve (AUC) of the ITT; H) non-fasted plasma cholesterol; I) FPLC profiles; J) triglycerides at the time of sacrifice; K) liver/body weight ratio; L) hepatic triglycerides, and M) hepatic total cholesterol at the end of the dietary intervention. Data are presented as mean ± SEM; $N = 8$ for each group. Statistically significant differences are indicated as $*p < 0.05$; $**p < 0.01$, $***p < 0.001$.

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Table 1. Gene expression in control and GOS-fed mice.

Genes	Control	GOS
Liver		
Hmgcoar	1.00 ± 0.44	0.80 ± 0.32
Cyp7a1	1.00 ± 0.32	0.67 ± 0.41
Cyp8b1	1.00 ± 0.31	0.62 ± 0.21**
Cyp27	1.00 ± 0.09	1.02 ± 0.15
Srebp1c	1.00 ± 0.27	1.02 ± 0.48
Ldlr	1.00 ± 0.18	0.81 ± 0.16
Srebp2	1.00 ± 0.10	0.82 ± 0.17*
Proximal intestine		
Apo C3	1.00 ± 0.26	0.93 ± 0.18
GLP-1	1.00 ± 0.11	1.66 ± 0.46***
Mttp	1.00 ± 0.19	1.09 ± 0.34
Distal intestine		
Asbt	1.00 ± 0.27	1.45 ± 0.34
Fgf15	1.00 ± 0.31	0.74 ± 0.28
White adipose tissue		
TNF α	1.00 ± 1.23	0.68 ± 0.87
UCP1	1.00 ± 0.35	1.14 ± 0.31

Tissues were excised during sacrifice and stored at −80 °C. Quantitative real-time PCR was performed as described in Experimental Section. Each gene is expressed as a ratio to the housekeeping gene 36B4 and further normalized to the expression level of the respective control group. Data presented as means ± SD; at least $N = 8$ for each group. Statistically significant differences are indicated as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

of feces, cholesterol and dihydroxy (DiH)-cholesterol remained unchanged. In contrast, coprostanol, a major bacteria-derived product, was substantially higher in GOS-fed mice (+370%, Figure 3B, $p < 0.05$) translating into an overall significant increase in total fecal neutral sterol excretion in GOS-supplemented mice compared to the control group (+50%, Figure 3B, $p < 0.05$). On the other hand, the excretion of bile acids, another major route for cholesterol disposal from the body, was significantly reduced in the feces of GOS-fed mice (−38%, Figure 3C, $p < 0.01$). Consistent with this suggestion of a decreased steady state bile acid synthesis, *mRNA* expression of genes that encode for two key enzymes involved in hepatic bile acid synthesis, namely cholesterol 7α-hydroxylase (*Cyp7A1*) and sterol 12-alpha-hydroxylase (*Cyp8b1*), was lower in the GOS group (Table 1). In addition to changes in mass, we also observed alterations in bile acid profiles (Figure 3D) with almost proportionate decreases in cholic acid (CA, −50%, $p < 0.05$), α-muricholic acid (α-MCA, −54%, $p < 0.05$) and deoxycholic acid (DCA, −40%, $p < 0.01$), while hyodeoxycholic acid excretion was substantially higher in GOS-fed mice (HDCA, +260%, $p < 0.01$). In plasma, total bile acids were moderately however, not significantly increased in GOS-fed mice (Figure 3E, $p = 0.09$). Relatively higher proportions of ursodeoxycholic acid (UDCA, +90%, $p < 0.05$) and β-muricholic acid (β-MCA, +60%, $p < 0.05$) were present in the GOS group compared to controls (Figure 3F). Taurocholic acid (TCA, −65%, $p < 0.01$) was present in a lower proportion in plasma of GOS-supplemented mice. HDCA was detectable in the plasma of the GOS group in appreciable amounts, whereas it was minimal in the control group.

2.4. Dietary Supplementation of GOS Delayed the Appearance of Enterally Administered Fat into the Blood

In order to investigate whether GOS feeding had a potential impact on fat absorption in the intestine we performed an oral fat tolerance test and assessed the appearance of enterally administered fat into the plasma. In GOS-supplemented mice, triglyceride appearance in plasma was evidently reduced at the 2 and 4 h time points suggestive for a decreased intestinal fat absorption rate (**Figure 4**C). The intestinal *mRNA* expression of genes encoding for lipid transporters, as well as factors contributing to chylomicron production such as microsomal triglyceride transfer protein (Mttp) and apolipoprotein C3 (ApoC3) remained unchanged (Table 1).

2.5. GOS Supplementation Shifted the Composition of Cecal Microbiota

Illumina HiSeq 16S rRNA gene sequencing yielded 3 325 258 (Min: 51 558; Max: 56 339; Median: 175 285; Mean: 207 828.625; Std. dev.: 151 030.39) reads that passed the quality check and could be assigned to 278 OTUs from 59 bacterial genera. Genus level taxa detected at an average relative abundance above 0.001 in at least one of the treatment groups are listed in Table S2, Supporting Information. On average, the three most abundant genera were *Allobaculum*, *Faecalibaculum*, and uncultured bacterium from *Bacteroidales S24-7*. The combined relative abundance of these taxa comprised more than 56% of all detected taxa. GOS feeding resulted in significantly higher levels of Actinobacteria, specifically *Bifidobacterium* and *Parvibacter*, Betaproteobacteria: *Parasutterella*, as well as *Akkermansia* and uncultured genus within family *Erysipelotrichaceae* (FDR<0.05). GOS supplementation was associated with a significant reduction in Firmicutes taxa, specifically within Clostridia, mainly in families *Lachnospiraceae*, *Ruminococcaceae*, and *Peptostreptococcaceae*, as well as genera *Olsenella*, *Alistipes*, *Faecalibaculum*, and *Bilophila*. Differentially abundant taxa in GOS and control groups identified in LefSe biomarker discovery analysis with a significance cutoff $p < 0.01$ are summarized in **Figure** 5A.

Overall fewer genus level taxa were detected in the GOS treatment group animals than in the controls (observed species: 44 vs 53 respectively; FDR = 0.004). A significant difference was also detected when Chao1 species richness scores were compared (Chao1: 59 vs 90 respectively, FDR = 0.001). GOS and control group animals also differed in their microbiota diversity (PD Whole Tree scores: 4.5 vs 5.0 respectively; FDR = 0.019), but not when Shannon diversity indices were compared (3.5 vs 4.2 respectively; FDR = 0.094), indicating that the control diet induced microbial community was more phylogenetically diverse (distant) than the community supported with GOS-supplemented diet. Genus level based PCA analysis revealed a strong effect of diet on the cecal microbial communities as indicated by the clear separation of animals from different treatment groups (data not shown), and the results were similar when PCoA analysis was used with either weighted and unweighted unifrac distances data (Figure S1, Supporting Information). ANOSIM analysis indicated significant differences between treatment groups when

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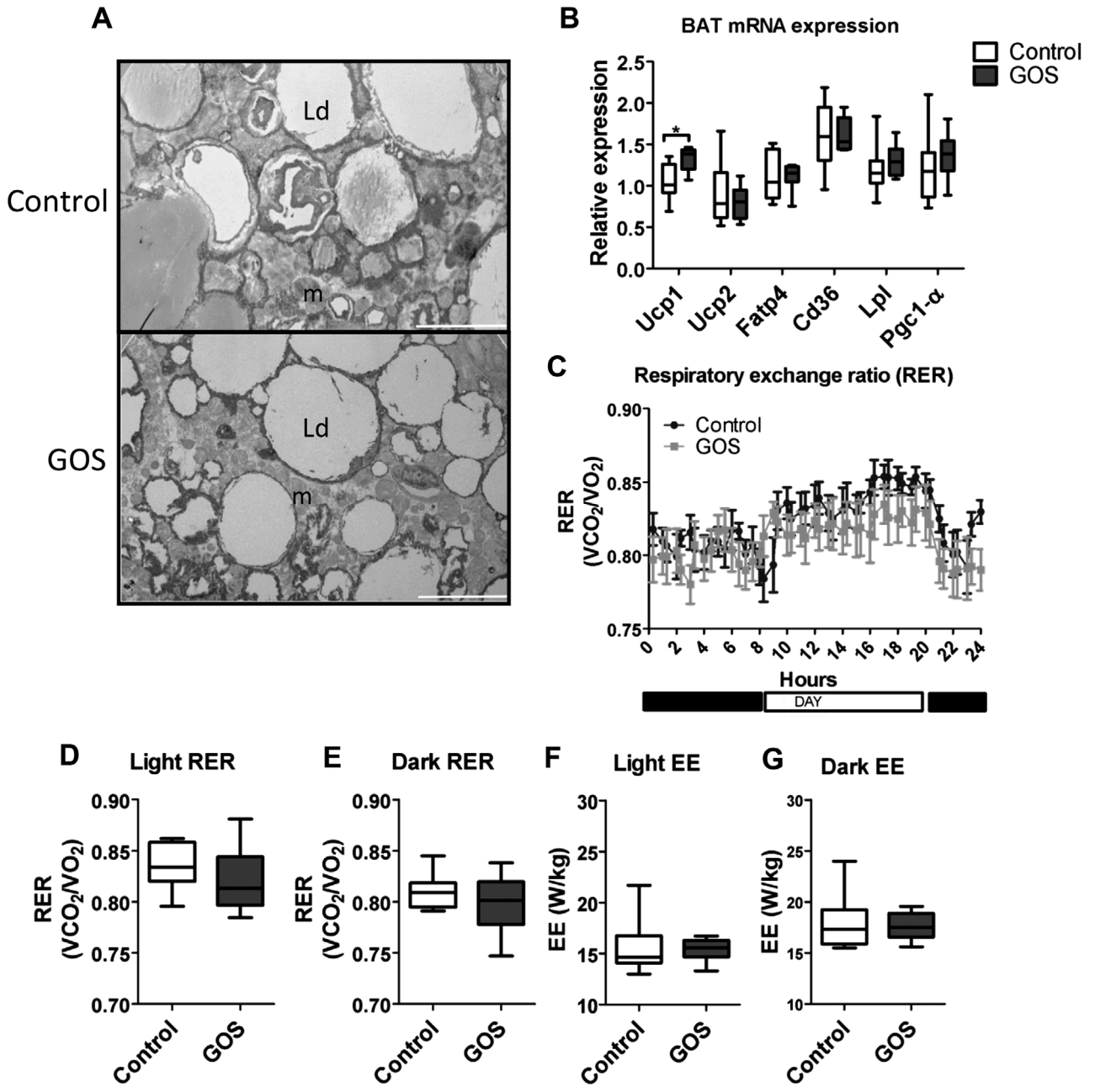

Figure 2. GOS supplementation does not alter energy metabolism. A) Representative images from electron microscopy of brown adipose tissue (BAT). Ld: lipid droplet, m: mitochondria, bar = 10 µm; B) *mRNA* expression in BAT; C) respiratory exchange ratio (RER); D) RER during light hours; E) RER during dark hours; F) energy expenditure (EE) during light hours; G) EE during dark hours. Data are presented as mean ± SEM; $N = 8$ for each group. Statistically significant differences are indicated as $^*p < 0.05$.

comparing weighted (test statistic = 0.220; FDR = 0.023) and unweighted (test statistic = 0.880; FDR = 0.001) unifrac distances. Diet explained 42.6% variation in the microbiota, with vector position indicating that among other taxa, the health benefiting *Bifidobacterium* and *Akkermansia* were associated with GOS treatment (Figure 5B). Furthermore, Spearman correlation analysis identified strong positive correlations between *Bifidobacterium*, *Parvibacter*, *Olsenella*, and an uncultured genus within the *Ersipelotrichaceae* with intestinal proglucagon expression and fecal hyodeoxycholic acid. In addition, several other microbial taxa positively correlated with fecal deoxycholic acid (Figure 5C).

3. Discussion

The results of the present study demonstrate that supplementing a "Western" type diet with β-GOS for 15 weeks led to reduced

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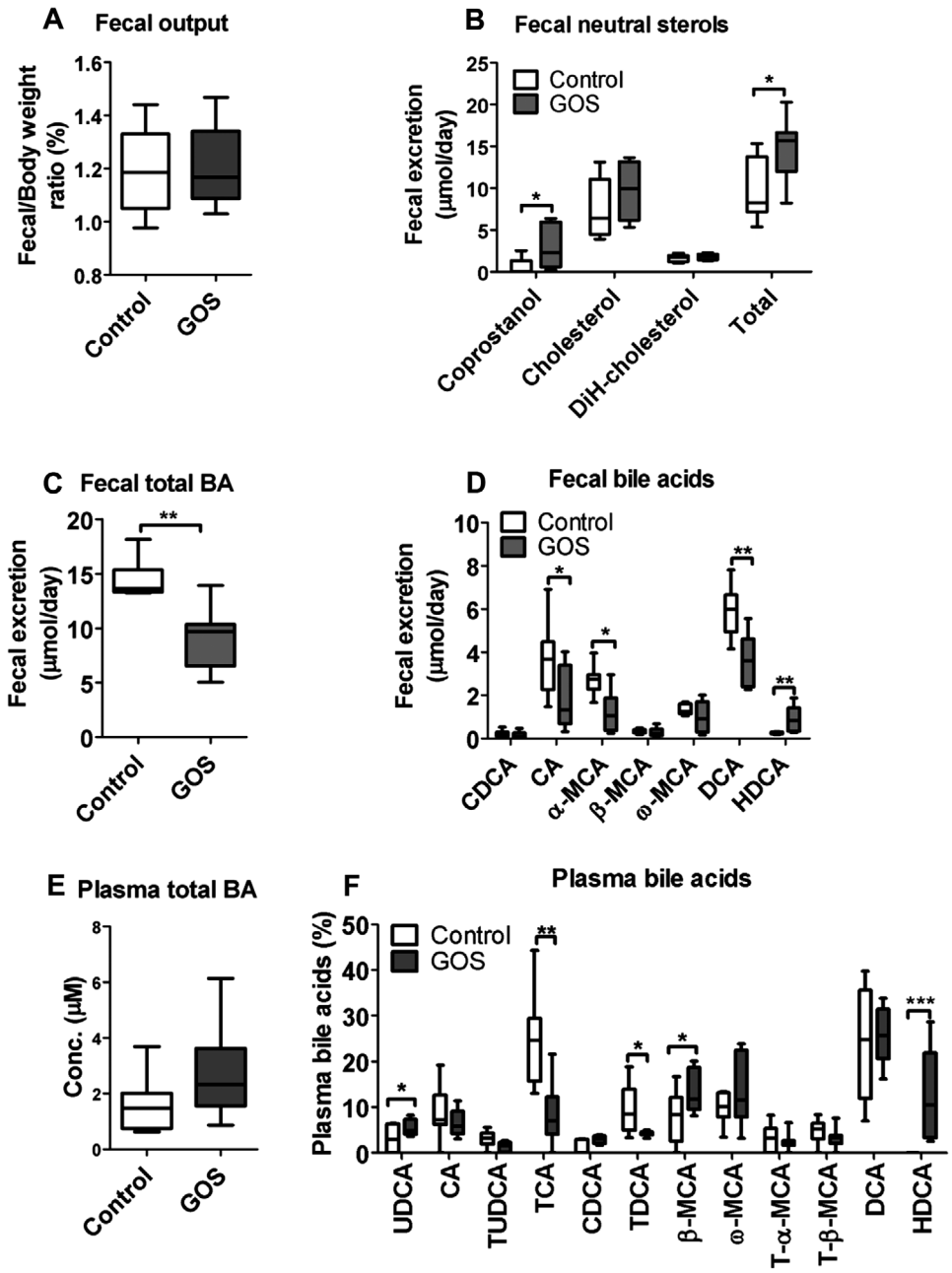

Figure 3. GOS supplementation alters fecal sterol excretion. A) 24 h fecal mass output to body weight ratio at the end of the dietary intervention; B) fecal neutral sterol excretion; C) total fecal bile acid (BA) excretion; D) fecal excretion rates of individual bile acid species; E) plasma total BA; F) plasma bile acid profiles. Data are presented as mean ± SEM; $N = 8$ for each group. Statistically significant differences are indicated as $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

body weight gain and subsequently decreased adiposity in mice. Development of insulin resistance was also reduced, conceivably as a consequence of reduced weight gain and adiposity. In addition, GOS-fed mice had a less atherogenic plasma lipid profile. GOS feeding decreased the intestinal fat absorption rate and increased intestinal GLP-1 expression. Combined these data, if confirmed in humans, support the use of GOS as a food supplement in the prevention or treatment of metabolic syndrome related disease.

Increase in consumption of "Western" type diets has accelerated the development of obesity,[11] and the impact of supplementing GOS as dietary fibers has, to the best of our knowledge, not been studied before. Dietary fibers have been reported to enhance satiety perception as well as to delay hunger onset. Satiety signaling hormones such as glucagon-like peptide (GLP-1) have been identified to influence satiation. GLP-1 is expressed in L-cells of the proximal ileum and colon and was reported to reduce food intake and delay gastric emptying.[12] Past studies in

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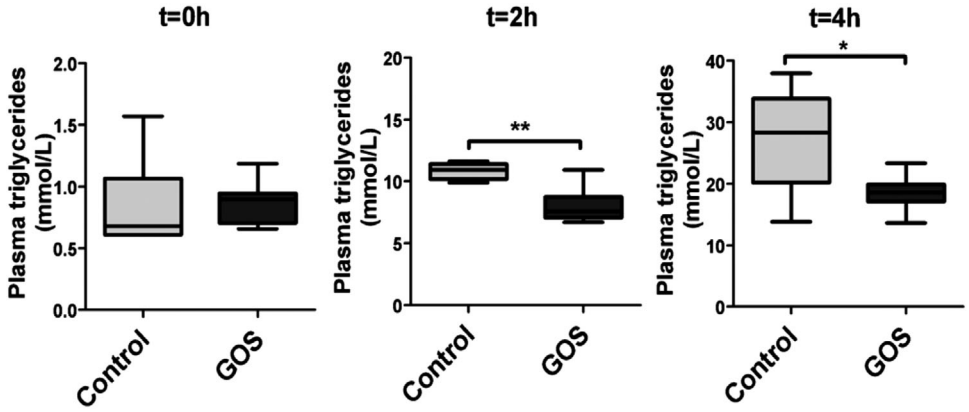

Figure 4. Plasma triglycerides during an oral fat absorption test. A) 0 h, B) 2 h, C) 4 h. Data are presented as mean ± SEM; $N = 8$ for each group. Statistically significant differences are indicated as $*p < 0.05$; $**p < 0.01$.

β-GOS-fed rats have shown increased colonic expression of GLP-1.[13] The secretion of such hormones can be regulated by a variety of molecules with signaling properties. Particularly, SCFA and bile acids such as hyodeoxycholic acid were shown to trigger the release of satiety hormones including GLP-1.[14,15] Hyodeoxycholic acid was highly increased in feces by GOS administration in the present study lending further plausibility to the proposed mechanism via a shift in bile acid composition. Consistent with these findings we observed a decreased body weight gain in the GOS group together with an increased GLP-1 expression. We did not observe a decreased food intake in GOS treated mice, but a more direct interaction of GLP-1 with specific tissues such as the pancreas or indirectly via liver, adipose tissue, or central nervous system circuits cannot be excluded.[16,17] Our analysis further revealed that GOS feeding in animals in the presence of higher dietary fat could reduce the rate of intestinal fat absorption. This effect could potentially also be attributed to GLP-1, since it was shown that gut-derived GLP-1 can decrease intestinal chylomicron production via a gut–brain axis.[18]

Dyslipidemia is one of the major risk factors for cardiovascular disease. Dyslipidemia associated with obesity is characterized by increased triglycerides, increased LDL cholesterol, and decreased HDL cholesterol.[19] A moderate shift in the lipoprotein profile with decreased LDL cholesterol was found in GOS-fed animals compared to controls. The results indicate that GOS supplementation could prove useful in helping to normalize a proatherogenic lipoprotein profile in addition to, for example, statins, the current mainstay of medication in the cardiovascular field.

Interestingly, GOS supplementation led to significant shifts in fecal sterol excretion. While total neutral sterol excretion was higher in GOS-fed animals compared to the control group, the fecal excretion of bile acids was almost proportionally decreased. The increase in fecal coprostanol in GOS-fed animals likely reflected a shift in intestinal bacterial populations induced by GOS since coprostanol is a product of bacterial metabolism. Bacterial enzymes play an important role in forming coprostanol by reducing the double bond between carbon 5 and 6 of cholesterol molecules[20] and also in converting primary into secondary bile acids. Total bile acids in feces were reduced in GOS-fed mice mirrored by the downregulation of hepatic *Cyp7a1* and *Cyp8b1* mRNA expression in GOS-fed mice, while the fecal bile acid profile reflected a substantial shift in different species in response to dietary GOS. Particularly remarkable was the high level of hyodeoxycholic acid in plasma and feces of GOS-fed mice. In hamsters, dietary hyodeoxycholic acid was shown to decrease cholesterol absorption thereby lowering plasma LDL-cholesterol levels and increasing fecal cholesterol excretion.[21] We observed congruent physiological changes in our present mouse study in the GOS group.

Alterations in gut microbial populations are known to contribute to changes in host metabolism and to dysbiosis in particular with respect to the development of obesity.[4,22] In the present study significant GOS-induced changes in cecal microbial populations were found that are in agreement with previous studies utilizing different GOS preparations.[7,23,24] A marked increase was observed in the relative abundance of *Bifidobacterium* and *Akkermansia* in the GOS group. Both of these are known to have beneficial effects on host metabolism.[25,26] The bifidogenic effect of GOS was consistent with what was observed in individuals with obesity,[25] however, in these GOS supplementation had no effect on body weight and insulin sensitivity. It was recently shown in elegant studies that *Akkermansia* improves obesity and glycemic control, although these effects might be strain-dependent and thus not in detail confirmable with the resolution of our study.[27,28] *Bifidobacterium* on the other hand mostly generates acetate and lactate—consistently observed also in our study—which acidify the intestinal environment and potentially restrict growth of pathogenic bacteria and improve mucosal barrier function.[29,30] High-fat feeding causes reduced growth of *Bifidobacterium* species.[31] However, our study showed that supplementing a Western-type diet with GOS still potently stimulates *Bifidobacterium* growth. Gut microbial derived metabolites can influence various metabolic parameters.[15,32] Our analyses also revealed significant correlations of bacterial species with various bile acid species, total fecal neutral sterol excretion and intestinal GLP-1 expression. Specifically, growth of *Bifidobacterium*, *Parvibacter*, *Olsenella*, and *Ersipelotrichaceae* showed significant correlation with GLP-1. Given that *Bifidobacterium* is associated with the generation of acetate, GOS-feeding could potentially stimulate such a mechanism via the acetate-mediated GLP-1 secretion pathway.[33,34] In the interpretation of the microbiota-related results we feel that a potential limitation of our study

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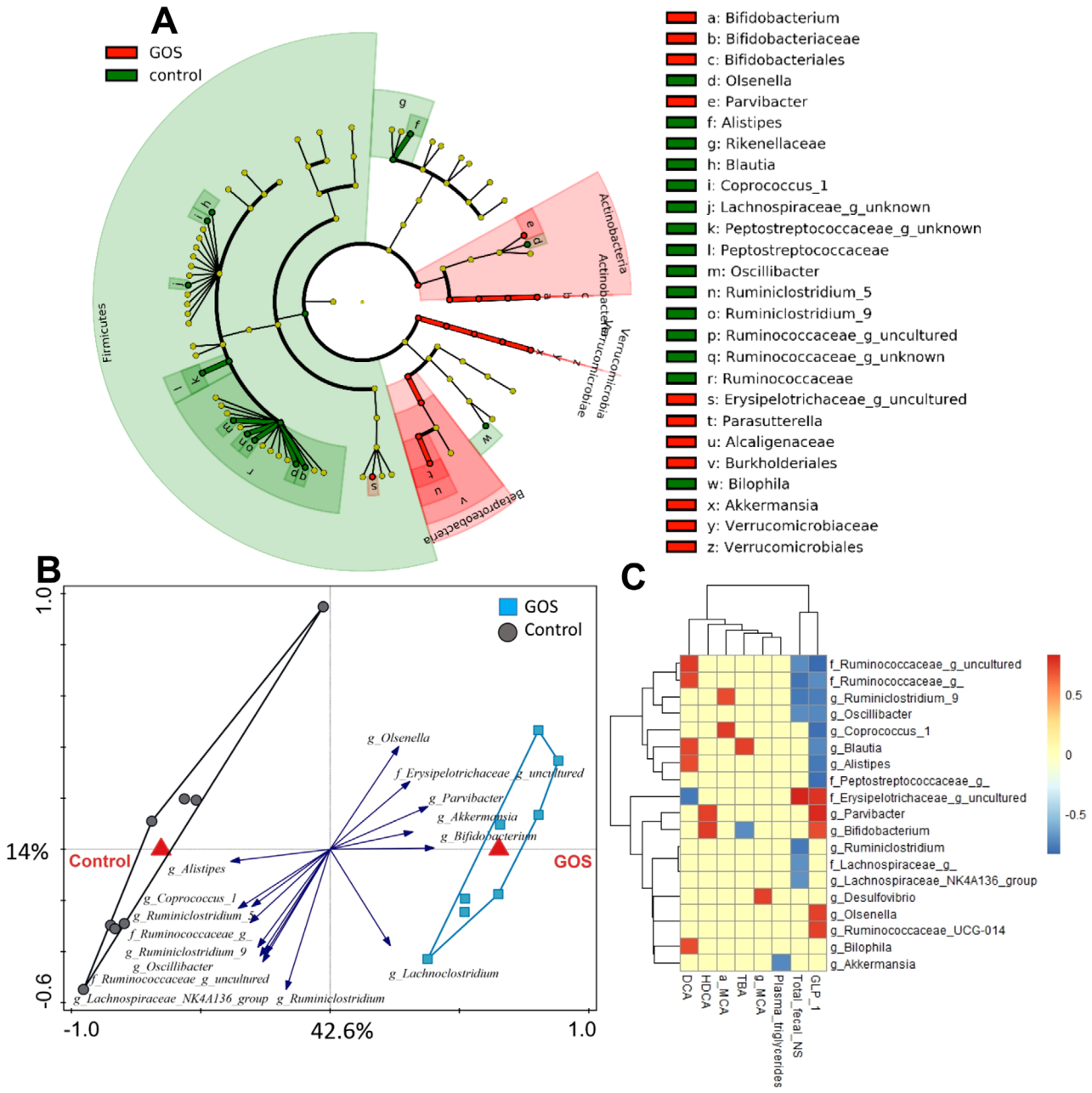

Figure 5. GOS induces a favorable shift in the cecal microbiota composition. A) LefSe cladogram showing differentially abundant phylum, class, order, family, and genus level taxa between GOS and control treatment groups; B) RDA triplot showing spatial distribution of cecal microbiota samples color-coded and enveloped by treatment group. The fifteen best fitting genus level taxa are projected on the graph. The percentage of total variance explained by first (constrained) and second (unconstrained) canonical axes are included indicating a strong effect of the diet. C) Heat map of correlations between relative abundance of genus level microbial taxa and various metabolic parameters. Red boxes indicate positive and blue negative correlations. Correlations that did not pass the cutoff of $p < 0.05$ and the correlation threshold = 0.7 are indicated with yellow boxes. Abbreviations: GLP-1 (Glucagon-like peptide-1), NS (neutral sterol), *g*-MCA (*ω*-muricholic acid), TBA (total bile acids), a-MCA (*α*-muricholic acid), HDCA (hyodeoxycholic acid), and DCA (deoxycholic acid).
Note: When the taxonomic assignment was not available at genus level classification, the lowest classifiable taxonomy assignment was used instead and unidentified genus was indicated with "g_g".

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needs to be pointed out. We chose for the GOS-containing and the control diet to have similar energy densities and replaced corn starch with GOS instead of comparing different fibers. Therefore, this experiment does not allow to clearly distinguish between effects specific to GOS from differences that could be ascribed to presence versus absence of fibers. Further studies would be required to identify bacterial strains responsible for the GOS-specific effects on host metabolism.

With respect to the relevance of our findings for the human situation, thus far, to the best of our knowledge, only one intervention trial is available investigating the effect of β-GOS as used in our study on insulin sensitivity and weight gain in a limited number of prediabetic subjects. No significant impact on both of these parameters was seen, however, the fecal abundance of *Bifidobacterium* species increased significantly.[9] Interestingly, fecal SCFA remained unaltered, too, indicating that the amount of GOS used (15 g d^{-1}) might have been too low to produce significant physiological benefits. On the other hand, no gastrointestinal side effects occurred. More work appears to be required in this respect. In humans, dosing could represent a problem, since the occurrence of loose stools due to decreased fat absorption could conceivably result in reduced compliance. Another study, using α-GOS though reported that in young healthy adults, fasting glucose levels increased, while OGTT results were not impacted after a 2-week intervention.[10] Potential physiological effects of the different chemical structures between α- and β-GOS remain to be explored. In addition, it has been shown that different subjects can have a diametrically different metabolic response to the same food[35]; thus, to efficiently make use of, for example, GOS in human nutrition, a personalized approach might be required.

In conclusion, we demonstrate that supplementing a Western-type diet with GOS reduced the rate of intestinal fat absorption and resulted in lower body weight gain, less adiposity, reduced insulin resistance and a less atherogenic plasma lipid profile. Although further studies in humans seem warranted to substantiate these effects, our work indicates that GOS supplements could offer an attractive option to reduce metabolic syndrome-related disease risk, one of the major health burdens of our times.

4. Experimental Section

Animal Experimental Design: Male C57BL/6OlaHsd mice were obtained from Harlan (Horst, The Netherlands). At the start of the dietary intervention all mice were 9 weeks of age. All mice were housed individually in a light- and temperature-controlled facility (12 h light-dark cycle, 12 °C). All animal experimentations were approved by the Committee of Animal Experimentation at the University of Groningen (permit # 6905) and performed in accordance with the Dutch National Law on Animal Experimentation (Wod) as well as international guidelines on animal experimentation. Vivinal GOS powder (FrieslandCampina, The Netherlands) was generously provided by Dr. Henk Schols (Wageningen University & Research, The Netherlands). The product contained 70% w/w β-GOS (main structural element: β-D-Galp-(1→4)), 24% w/w lactose, and 6% w/w monosaccharides (glucose and galactose). The control high-fat baseline diet (27% fat; energy 21.3 MJ kg^{-1}) was from Ssniff diets (Soest, Germany) and GOS-supplemented diet (27% fat; energy 21.3 MJ kg^{-1}) was obtained by replacing an equal amount of corn starch with GOS (7% w/w, for detailed composition see Table S1, Supporting Information). Animals were fed ad libitum with control ($n = 8$) and GOS ($n = 8$) supplemented diets for a period of 16 weeks. Animals were weighed every week. Food intake was measured after 8 and 15 weeks. At the end of the dietary intervention the gastrointestinal tract, liver, and adipose tissues were excised, collected, and stored at −80 °C until later analysis. Power analysis indicated that with an assumption of 80% power and a two-sided α significance of 0.05 the study was sufficiently powered with group sizes of $n = 8$ to detect the observed difference in body weight as outcome parameter. The study was repeated with an identical set-up to confirm the obtained results as well as to add determinations such as indirect calorimetry (see below).

Analysis of Plasma and Liver: Blood samples were collected by heart puncture at the time of termination. Plasma was isolated and aliquots were stored at −80 °C until further analysis. For lipoprotein fraction analysis, plasma samples were pooled and subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Health, Uppsala, Sweden) as described previously.[36] Bligh and Dyer procedure was used to extract lipids from liver homogenates which were then subsequently redissolved in water containing 2% Triton X-100 as described previously.[37] Commercially available reagents (Roche, Diagnostic, Basel, Switzerland) were used to measure plasma and hepatic total cholesterol and triglycerides.[38]

Fecal Mass Sterol, Fatty Acids, Bile, and Short-Chain Fatty Acids Measurements: Fecal samples were obtained from the bedding following collection over a 24 h period. The samples were dried, weighed, and ground. 50 mg of ground feces was used for extraction of neutral sterols and bile acids. A mixture of acetyl chloride and trimethylsilytate with pyridine, N,O-bis(trimethylysilyl)trifluoroacetamide and trimethylcholorosilane was used for methylating bile acids. Fecal neutral sterols and bile acids were then measured using gas–liquid chromatography as published earlier.[37] Cecal short-chain fatty acids and lactate were determined at the time of sacrifice by gas–liquid chromatography as described[39] after extraction from 50 mg of cecum content as detailed previously.[40]

Indirect Calorimetry and Body Composition Analysis: At the end of the dietary intervention body composition was measured using the MiniSpec LF90 TD-NMR analyzer (Bruker BioSpin, Billerica, MA, US). Each animal was placed inside the restraint tube unanesthetized and without impairing respiration. After body composition measurements animals were returned to their cage. 1 week before sacrifice body composition was analyzed in another cohort using a Minispec whole body composition analyzer (Bruker). Respiratory exchange ratio (RER) and energy expenditure (EE) were determined using a comprehensive laboratory animal monitoring system (TSE Systems GmbH, Bad Homburg, Germany).

Glucose Tolerance and Insulin Tolerance Tests: Intraperitoneal glucose tolerance test was conducted at the end of the dietary intervention period by intraperitoneal administration of 2.5 g glucose per kg body weight.[41] The animals were fasted for 6 h prior to the test. For intraperitoneal insulin tolerance tests, animals were fasted for 4 h prior to the intraperitoneal injection of insulin (Novo Nordisk, Denmark) at 0.75 unit kg^{-1} body weight.

Electron Microscopy: At the time of sacrifice, small pieces (≈3–5 mm) of brown adipose tissue were cut and fixed in 0.1% glutaraldehyde (GA) in 0.1 M sodiumcacodylate buffer. Following overnight fixation at 4 °C, tissue was embedded in EPON using standard procedures.[42] Images were taken using 3400× magnification in a transmission electron microscope (CM100; FEI Company, The Netherlands) at 80KV.

Assessment of Fat Absorption Kinetics: At the end of the dietary intervention, mice were fasted overnight and then given an intraperitoneal injection with poloxamer 407 (P407, 1 g kg^{-1} body weight), which completely inhibits the catabolism of apolipoprotein B-containing lipoproteins. Immediately after, an intragastric load of 150 µL olive oil was given by gavage. Subsequently, blood samples were collected into heparinized tubes from the retro-orbital plexus at time 0, 2, and 4 h. Plasma triglycerides were measured using the reagents mentioned above and since triglyceride catabolism is inhibited by P407, changes in plasma levels are a reflection of absorption rates.

Quantitative Real-Time PCR Gene Expression Analysis: Total RNA extraction was performed using TriReagent (Sigma). Nanodrop ND-100UW-vis spectrometer (NanoDrop Technologies Wilmington DE) was used to measure the RNA concentration. cDNA was synthesized with one µg of RNA using Invitrogen (Carlsbad CA) reagents. ABI Prism 7700 machine (Applied Biosystem, Damstadt Germany) was used to perform real time

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PCR using primers synthesized by Eurogentec (Seraing, Belgium). To calculate the individual relative *mRNA* expression, 36B4 gene expression was used as a housekeeping gene, and values were further normalized to the relative expression of the individual control group.[38]

Glp-1 Determination: Glp-1 was measured by ELISA in plasma that was immediately frozen after collection without addition of a protease inhibitor, following the manufacturer´s instruction (EMD Millipore, St. Louis, MO, USA).

Microbiota Analysis: Total bacterial DNA was extracted from 0.01 to 0.1 g of cecal contents using the double bead-beating procedure as previously described.[43] Briefly, the V4 regions of 16S ribosomal RNA (rRNA) genes were PCR amplified with uniquely barcoded primer pair: 515F(5′-GTGCCAGCMGCCGCGGTAA)-806R(5′-GGACTACHVGGGTWTCTAAT), and the barcoded PCR products were then purified and pooled into an amplicon library containing 100 ng of each sample. The pool was adjusted to 100 ng μL^{-1} final concentration and sent for adapter ligation and Illumina HiSeq2000 sequencing at GATC-Biotech, Konstanz, Germany.[43] The 16S rRNA gene sequencing data was analyzed using the NG-Tax analysis pipeline[44] with standard parameters and SILVA_128_SSU 16S rRNA gene reference database (https://www.arb-silva.de/) to assign taxonomy.[45]

Statistics: Statistical analysis for the physiological parameters was performed using GraphPad Prism software (San Diego, CA). All data are presented as means ± SEM. Statistical significance was assessed using the Mann–Whitney *U*-test and was assigned to $p < 0.05$. With respect to the microbiome, microbiota alpha diversity indices (Shannon, Chao1, and PD Whole Tree) were calculated on rarefied read data (cutoff = 50 000 reads per sample) and compared between treatment groups using a nonparametric two sample t-test with Monte Carlo permutations in QIIME.[46] Weighted and unweighted unifrac distances were calculated and compared using ANOSIM test (QIIME). Differentially abundant taxa between treatment groups were identified using Kruskal–Wallis analysis (QIIME). Unconstrained (PCA) and constrained redundancy analysis (RDA) was carried out in Canoco5, with significance assessed using a permutation test.[47] Resulting p values in the RDA analysis were corrected for multiple comparisons using FDR method with significance cutoff set at FDR < 0.05. Biomarker taxa associated with different dietary treatments at significance cutoff $p < 0.01$ were identified and visualized using LefSe modules incorporated into Galaxy.[48] Spearman correlations were calculated in R (version 3.4.3) to evaluate associations between the relative abundance of different microbial genera and levels of metabolic biomarkers in plasma and feces. Correlations passing the threshold ct = ± 0.7 and the significance cutoff of $p < 0.05$ were visualized using the pheatmap function in R.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

R.H.M. and F.L. contributed equally to this work. R.H.M., design of the study, data acquisition, analysis, and interpretation of data, drafting the article; F.L., data acquisition and analysis, critical revision of the manuscript; K.B., data analysis and critical revision of the manuscript; M.A.M.L., data acquisition; H.S., critical revision of the manuscript for important intellectual content; H.J.V., interpretation of data, critical revision of the manuscript. U.J.F.T., conception and design of the study, interpretation of data, drafting the article. All authors read and approved the final manuscript.

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adipose tissue, galactooligosaccharides, lipid absorption, microbiota, prebiotics

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