Interplay between dietary fibers and gut microbiota for promoting metabolic health

Mistry, Rima

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Galacto-oligosaccharides supplementation decreases obesity and improves insulin sensitivity in mice fed a Western-type diet

Rima H. Mistry, Fan Liu, Klaudyna Borewicz, Mirjam A. M. Lohuis, Hauke Smidt, Henkjan J. Verkade, Uwe J.F. Tietge

Manuscript in preparation
Abstract

The incidence of metabolic syndrome related disease is rapidly increasing. Accumulating evidence points towards a critical role of the gut microbiota as modifier of metabolic disease development. Dietary fibers such as galacto-oligosaccharides (GOS) have been shown to stimulate the growth of beneficial bacteria. In the present study, C57BL/6 mice were fed Western-type diet for 15 weeks with or without GOS (10% w/w) to assess the impact on obesity and on glucose and lipid metabolism. GOS feeding reduced body weight gain compared to controls (-8%, p<0.01), including lower accumulation of epididymal (-12%, p<0.05) and perirenal (-29%, p<0.01) fat. GOS feeding decreased plasma cholesterol (-20%, p<0.05), mainly due to lower levels of low-density lipoproteins. Interestingly, GOS consumption significantly decreased the appearance of enterally administered fat into the blood, suggestive for a delayed fat absorption (p<0.01). GOS increased intestinal GLP-1 mRNA expression (+66%, p<0.001). Fecal neutral sterol excretion increased (+50%, p<0.05), while fecal bile acid excretion decreased (-38%, p<0.01) in GOS-fed mice. Bile acid profiles indicated substantial compositional differences in the GOS group, namely lower cholic (-50%, p<0.05), α-muricholic (-54%, p<0.05), and deoxycholic acid excretion (-40%, p<0.01), whereas hyodeoxycholic acid excretion increased (+260%, p<0.01). Substantial, conceivably beneficial changes in microbiota composition were recorded upon GOS feeding. In conclusion, GOS supplementation to mice fed a Western-type diet improves body weight gain, dyslipidemia and insulin sensitivity. Our data support the therapeutic potential of GOS supplementation for individuals at risk of developing metabolic syndrome.
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. 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. 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. A significant increase in pathogenic and a reduction in beneficial microbial strains has been associated with inflammation, obesity and metabolic dysregulation.

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

Galacto-oligosaccharides (GOS) are dietary fibers derived from lactose using a β-galactosidase enzyme. GOS is a soluble fiber widely used for its potential to alter gut microbiota composition by stimulating growth of supposedly beneficial bacteria such as Bifidobacterium and Lactobacillus. Different varieties of GOS have been utilized in a limited number of clinical studies. 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. However, thus far the long-term effects of GOS on the development of obesity and insulin resistance have not been studied either in humans or in preclinical models. The present work aimed to investigate long-term metabolic effects of GOS supplementation to a Western-type diet in vivo in mice, including an evaluation of potential underlying mechanisms.

Materials and Methods

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 (12h light-dark...
Interplay between dietary fibers and gut microbiota for promoting metabolic health

cycle, 12°C). All animal experimentations were approved by the Committee of Animal Experimentation at the University of Groningen and performed in accordance with the Dutch National Law on Animal Experimentation (Wod) as well as international guidelines on animal experimentation. GOS powder was generously provided by Dr. Henk Schols (Wageningen University, The Netherlands). The product contained 70% GOS, 24% lactose and 6% monosaccharides (glucose and galactose). The control high-fat diet was obtained from Ssniff diets (Soest, Germany). Control high-fat diet (27% fat; energy 21.3 kcal/kg) contained 2.4% lactose, 0.6% glucose, 20% casein, 0.3% L-cystine, 1% vitamin mixture, 0.2% choline bitartrate, 3.5% mineral mixture, 2.5% soya bean oil, 24.5% lard, 37.845% corn starch, 7% carbohydrate mix (1:1 sucrose:maltodextrin), 0.005% FD&C blue dye and 0.15% cholesterol. GOS supplemented diet (27% fat; energy 20.3 kcal/kg) was obtained by replacing an equal amount of corn starch with GOS (7% GOS, 2.4% lactose, 0.6% glucose, 20% casein, 0.3% L-cystine, 10% vitamin mixture, 2% choline bitartrate, 3.5% mineral mixture, 2.5% soya bean oil, 24.5% lard, 30.845% corn starch, 7% carbohydrate mix (1:1 sucrose:maltodextrin), 0.05% FD&C blue dye and 0.15% cholesterol). 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.

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. Bligh and Dyer procedure was used to extract lipids from liver homogenates which were then subsequently redissolved in water containing 2% Triton X-100 exactly as published. Commercially available reagents (Roche, Diagnostic, Basel, Switzerland) were used to measure plasma and hepatic total cholesterol and triglycerides.

Fecal mass sterol, fatty acids and bile 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 (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane was used for methylating bile acids. Fecal neutral sterols and bile acids were then measured using gas-liquid chromatography as published earlier.\textsuperscript{12}

**Indirect calorimetry and body composition analysis**

One week before sacrifice body composition was analyzed using a Minispec Whole Body Composition Analyser (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.\textsuperscript{14} The animals were fasted for six hours prior to the test. For intraperitoneal insulin tolerance tests, animals were fasted for four hours prior to the intraperitoneal injection of insulin (Novo Nordisk, Denmark) at 0.75 unit/kg body weight.

**Assessment of fat absorption kinetics**

Mice were fasted overnight and then given an intraperitoneal injection with poloxamer 407 (1 g/kg body weight). 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 hours. Plasma triglycerides were measured using the reagents mentioned above.

**Quantitative real-time PCR gene expression analysis**

Total mRNA extraction was performed using TriReagent (Sigma). Nanodrop ND-100UW-vis spectrometer (NanoDrop Technologies Wilmington DE) was used to measure the mRNA concentration. cDNA was synthesized with one µg of mRNA using Invitrogen (Carlsbad CA) reagents. ABI Prism 7700 machine (Applied Biosystem, Damstadt Germany) was used to perform real time PCR using the synthesized cDNA and primers designed by Eurogentec (Seraing, Belgium). To calculate the individual relative mRNA expression, 36B4 gene expression is used as a housekeeping gene and further the values are normalized to the relative expression of the individual control group.\textsuperscript{13}
Microbiota analysis

Total bacterial DNA was extracted from 0.01-0.1g of cecal contents using the double bead-beating procedure as previously described. 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 final concentration and sent for adapter ligation and Illumina HiSeq2000 sequencing at GATC-Biotech, Konstanz, Germany. The 16S rRNA sequencing data was analyzed using the NG-Tax analysis pipeline with standard parameters and SILVA_111_SSU 16S rRNA gene reference database (https://www.arb-silva.de/) to assign taxonomy.

Statistics

Statistical analysis was performed using GraphPad Prism software (San Diego, CA). All data are presented as means ± SEM. Statistical differences between groups were assessed using the Mann-Whitney U-test. Statistical significance for all comparisons was assigned at p<0.05. Microbiota alpha diversity indices (Shannon, Chao1, and PD Whole Tree) were calculated on rarefied read data (cutoff = 50,000 reads/sample) and compared between treatment groups using a nonparametric two sample t-test with Monte Carlo permutations in QIIME. 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 in Canoco5 using the log transformed genus level relative abundance data with significance assessed using a permutation test at 499 permutations. 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. Spearman correlations were calculated in R (version 3.4.3) to evaluate associations between the relative abundance of different microbial genera and levels of coprostanol, cholesterol, DiH-cholesterol, total fecal NS, plasma cholesterol, plasma triglycerides and fecal bile acids: CDCA, α-MCA, DCA, CA, HDCA, β-MCA, and ω-MCA. Correlations passing the threshold ct = ±0.7 and the significance cutoff of p<0.05 were visualized using the pheatmap function in R.
Results

Dietary GOS supplementation improves body weight gain, dyslipidemia and insulin sensitivity

Prior to the dietary intervention both groups of animals were matched for age and body weight. Body weight was recorded weekly during the 15 weeks of dietary intervention with the Western-type diet with or without GOS. A significantly lower body weight gain (between 3-12%, Fig. 1A, p<0.01) was observed from the second week onwards, while food intake in both groups remained unchanged (Fig. 1B).

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

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

Tissues were excised during sacrifice and stored at -80°C. Quantitative real-time PCR was performed as described in methods. 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.

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%, Fig. 1C, p=0.055).
Figure 1: GOS supplementation improves 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 sacrifice; (K) liver/body weight ratio; (L) hepatic triglyceride and (M) hepatic total cholesterol at the end of the dietary intervention. Data are presented as mean ± SEM; at least N=8 for each group. Statistically significant differences are indicated as *p<0.05; **p<0.01, ***p<0.001.
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 (Fig. 1D). Glucose tolerance tests performed at the end of the dietary intervention indicated no differences between the groups (Fig. 1E). Insulin sensitivity, however, was improved in the GOS supplemented groups (Fig. 1F) with the area under the curve (AUC) being significantly lower in GOS fed animals (-20%, Fig. 1G, \( p<0.05 \)). Interestingly, GOS supplementation in mice increased the mRNA expression in the proximal intestine of glucagon-like peptide-1 (GLP-1) a gene encoding for an incretin hormone responsible for stimulating insulin secretion (+66%, Table 1, \( p<0.001 \)).

Western-type diet supplementation with GOS also changed the plasma and liver lipid profiles of the mice. At the end of the dietary intervention, plasma total cholesterol was lower in GOS-fed group (-20%, Fig. 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 (Fig.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%, Fig. 1J, \( p<0.05 \)). At week 15, GOS fed mice also showed a trend towards a lower liver/body weight ratio (Fig. 1K, \( p=0.06 \)). In GOS-fed mice, hepatic triglyceride levels tended to be lower (-33%, IL, \( p<0.06 \)), whereas hepatic cholesterol levels remained unchanged (Fig. 1M) compared to the control group.

**GOS supplementation does 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 (Fig. 2A). mRNA expression of several relevant genes remained unchanged (Fig. 2B). However, we detected a significant increase in uncoupling protein 1 (\( Ucp1 \)) which is responsible for expression of UCP1, a mitochondrial carrier protein of BAT involved in heat generation by disruption of the proton gradient during respiration (Fig. 2B, \( p<0.05 \)).
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=10µm; (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; at least N=8 for each group. Statistically significant differences are indicated as *p<0.05; **p<0.01, ***p<0.001.

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 are resting as well as during the night hours when the mice are active (Fig. 2C, 2D & 2E). Control and GOS-fed mice also showed similar energy expenditure (EE) during day and night hours (Fig. 2F & 2G). Thus, the increase in Ucp1 mRNA expression in BAT did not translate into a physiologically meaningful increase in energy metabolism.
Galacto-oligosaccharides supplementation decreases obesity and improves insulin sensitivity in mice fed a Western-type diet

GOS alters fecal neutral sterol and bile acids profiles

We investigated the role of GOS in 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 (Fig. 3A). In the neutral sterol profile 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%, Fig. 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%, Fig. 3B, p<0.05).

**Figure 3:** GOS supplementation alters fecal sterol excretion. (A) 24-hours 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; at least N=8 for each group. Statistically significant differences are indicated as *p<0.05; **p<0.01, ***p<0.001.
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%, Fig. 3C, p<0.01). Consistent with this suggestion of a decreased steady state bile acid synthesis, mRNA expression of 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 (Fig 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 (Fig. 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 (Fig. 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 GOS group in appreciable amounts, whereas it was minimal in the control group.

**Dietary supplementation of GOS delays 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 two and four-hour time points suggestive for a decreased intestinal fat absorption rate (Fig. 4C). The intestinal mRNA expression of 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).

![Figure 4: Plasma triglycerides during an oral fat absorption test. (A) 0 hour (B) 2 hour (C) 4 hour. Data are presented as mean ± SEM; at least N=8 for each group. Statistically significant differences are indicated as *p<0.05; **p<0.01.](image-url)
Galacto-oligosaccharides supplementation decreases obesity and improves insulin sensitivity in mice fed a Western-type diet

GOS supplementation induces a favorable shift in the composition of cecal microbiota

Illumina HiSeq 16S rRNA gene sequencing yielded 3,325,258 (Min: 51,558; Max: 563,39; Median: 175,285; Mean: 207,828.625; Std. dev.: 151,030.39) high quality 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 S1.

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) Heatmap 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. 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”.

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 Fig. 5A.

Overall there were fewer genus level taxa 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 groups 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 (Supplemental Fig.1). ANOSIM analysis indicated significant differences between treatment groups when 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 (Fig. 5B). Furthermore, Spearman correlation analysis identified strong positive correlations between Bifidobacterium, Parvibacter, Olsenella
Galacto-oligosaccharides supplementation decreases obesity and improves insulin sensitivity in mice fed a Western-type diet

and Ersipelotrichaceae with intestinal GLP-1 expression and fecal hyodeoxycholic acid. In addition, several other microbial species positively correlated with fecal deoxycholic acid as illustrated in the form of a heat map in Fig. 5C

Discussion

The results of the present study demonstrate that supplementing a “Western” type diet with GOS for 15 weeks reduces body weight gain and subsequently lowers adiposity in mice. Insulin sensitivity was also improved, conceivably as a consequence of the 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.

In recent years the incidence of obesity has seen a remarkable increase. Although the development of obesity involves a complex interaction between genetics and lifestyle, it is believed that the increasing consumption of “Western” type diets has accelerated this process. Supplementation of dietary fibers such as GOS under such circumstances and its potential impact on development of obesity has 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. 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. 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. Our analysis further revealed that GOS feeding in animals in the presence of higher dietary fat could substantially 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 brain-gut axis. It is unclear whether the decreased rate of intestinal fat absorption merely represents a kinetic phenomenon,
with an efficient absorption of dietary fat, or an actually quantitative malabsorption of dietary fat. A fecal fat balance would have made it possible to discriminate between these two possibilities, but this had not been included in the design of the study.

Obesity is also one of the main causes of the development of metabolic dysregulation which includes dyslipidemia, insulin resistance, type 2 diabetes and non-alcoholic fatty liver diseases (NAFLD, all major metabolic risk factors for cardiovascular disease \(^{35,36}\). Commonly, dyslipidemia associated with obesity is characterized by increased triglycerides, increased LDL cholesterol, and decreased HDL cholesterol.\(^ {37,38}\) In the present study GOS-fed mice had significantly lower plasma cholesterol and triglycerides at the end of the dietary intervention. A moderate shift in the lipoprotein profile with decreased LDL cholesterol was found in GOS-fed animals compared to the control group. Several processes are involved in uptake, transport and storage of cholesterol. Cholesterol is synthesized \textit{de novo} in the liver with HMG-CoA reductase serving as rate limiting enzyme, and LDL are taken up into cells via the LDL receptor. The present study demonstrates that HMGCoAR \textit{mRNA} expression was unchanged while plasma LDL-C was lower in GOS-fed animals despite the fact that the \textit{mRNA} expression of LDLR in the liver even tended to be decreased. This effect is different from the impact that statin therapy has, the current mainstay of medication in patients at cardiovascular risk. Statins namely increase hepatic LDLR levels and thereby promote LDL clearance, which leads to lower plasma LDL-C. If the results of our present study could be translated to the human situation, GOS supplements could prove useful in helping to normalize a proatherogenic lipoprotein profile in addition to statin therapy.

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. With respect to neutral sterols the main increase was found in fecal coprostanol in GOS fed animals, likely reflecting a shift in intestinal bacterial populations induced by GOS since coprostanol essentially is a product of bacterial metabolism. Bacterial enzymes form coprostanol by reducing the double bond between carbon 5 and 6 of cholesterol molecules.\(^ {39}\) Intestinal bacteria also play an important role in bile acid metabolism by converting primary into secondary bile acids. Total bile acids in feces were reduced in GOS-fed mice mirrored by the downregulation of hepatic \textit{Cyp7A1} and \textit{Cyp8b1} \textit{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. It has been reported that in hamsters dietary hyodeoxycholic acid decreases cholesterol absorption thereby lowering plasma LDL-cholesterol levels and increasing
fetal cholesterol excretion. 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. In the present study significant GOS-induced changes in cecal microbial populations were found that are in agreement with previous studies utilizing GOS. A marked increase was observed in *Bifidobacterium* and *Akkermansia* in the GOS group. Both of these are known to have beneficial effects on host metabolism. It was recently shown in elegant studies that *Akkermansia* improves obesity and glycemic control. *Bifidobacterium* on the other hand mostly generates acetate and lactate which acidify the intestinal environment and potentially restrict growth of pathogenic bacteria and improve mucosal barrier function. High-fat feeding causes reduced growth of *Bifidobacterium* species. 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. Our analyses also revealed significant correlations of bacterial species with various bile acid species, total fecal neutral 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.

In conclusion, we demonstrated that supplementing a Western-type diet with GOS reduces the rate of intestinal fat absorption and thereby results in lower body weight gain, less adiposity, improved insulin sensitivity 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.
References


Supplemental Figure & Table

**Supplemental figure 1:** PCoA analysis indicating separation of animals fed control and GOS diet based on unifrac distances. (A) weighted (B) unweighted
### Supplemental table 1: Relative abundance of bacterial taxa in the control and the GOS-fed group

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