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Mannose Alters Gut Microbiome, Prevents Diet-Induced Obesity, and Improves Host Metabolism

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SUMMARY

Mannose is an important monosaccharide for protein glycosylation in mammals but is an inefficient cellular energy source. Using a C57BL/6J mouse model of diet-induced obesity, we show that mannose supplementation of high-fat-diet-fed mice prevents weight gain, lowers adiposity, reduces liver steatosis, increases endurance and maximal O₂ consumption, and improves glucose tolerance. Mannose-supplemented mice have higher fecal energy content, suggesting reduced caloric absorption by the host. Mannose increases the Bacteroidetes to Firmicutes ratio in the gut microbiota, a signature associated with the lean phenotype. These beneficial effects of mannose are observed when supplementation is started early in life. Functional transcriptomic analysis of cecal microbiota revealed profound and coherent changes in microbial energy metabolism induced by mannose that are predicted to lead to reduced energy harvest from complex carbohydrates by gut microbiota. Our results suggest that the gut microbiota contributes to mannose-induced resistance to deleterious effects of a high-fat diet.

In Brief

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AUTHOR CONTRIBUTIONS

V.S. conceived, designed, and performed experiments and sequencing analysis; coordinated the study; and wrote the manuscript. J.S. and J.N. performed the animal experiments. J.E.A. conducted metabolic analysis (CLAMS, GTT, and ITT). D.A.S. developed GC-MS method for SCFA analysis. S.N.P. provided microbiome expertise and support for microbiome studies, and H.H.F. conceptualized the project and obtained funding. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND SOFTWARE AVAILABILITY

The microarray and RNA-seq data reported in this paper is available through NCBI's Gene Expression Omnibus (Edgar et al., 2002). The accession number is GEO: GSE110796.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and eight tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.08.064>.

A video abstract is available at <https://doi.org/10.1016/j.celrep.2018.08.064#mmc9>.

Sharma et al. show that mannose supplementation prevents adverse outcomes of high-fat diet when initiated early in life, not when provided later. Beneficial effects correlate with changes in gut microbial composition and could be partly attributed to lower energy harvest by the gut microbiota and host energy absorption.

Graphical Abstract

INTRODUCTION

Obesity reflects an imbalance between energy intake and expenditure, energy absorption, and utilization. Diet plays a dominant role in shaping the gut microbial community irrespective of genetic influence (Carmody et al., 2015) and responds very rapidly to alterations in diet (David et al., 2014). Fecal microbiome transplantation of germ-free (GF) mice established a relationship between the activities of the gut microbiota and obesity (Ridaura et al., 2013). Gut microbiome alterations impinge on specific host pathways, including energy regulation, systemic inflammation, enteroendocrine signaling, and gut barrier function (Bäckhed et al., 2004; Cani et al., 2008; Everard et al., 2013; Teperino et al., 2012; Turnbaugh et al., 2006).

Mannose is not a significant energy source in humans but is required for protein glycosylation (Stanley et al., 2017). It is also a therapy for congenital disorder of glycosylation patients with mutations in phosphomannose isomerase (MPI) (Niehues et al., 1998). Mannose is a safe and effective prophylactic for urinary tract infections (UTIs) caused by *Escherichia* (Kranj ec et al., 2014). A genome-wide transcriptomics study recently showed correlations between plasma mannose, BMI, and insulin resistance (Lee et al., 2016).

Here, we report that C57BL/6J mice weaned on a high-fat diet (HFD), supplemented with 2% (w/v) mannose in their drinking water, resist weight gain, have lower adiposity, reduced

liver steatosis, and improved glucose tolerance. These beneficial effects of mannose are mediated at least in part by changes in microbial composition and metabolism that reduced dietary energy harvest and absorption.

RESULTS

Mannose Reduces Weight Gain, Lowers Fat Mass, Reduces Liver Steatosis, and Improves Glucose Tolerance of HFD Mice

Three-week-old C57BL/6J male mice were randomly weaned on (1) normal diet (ND), (2) ND with 2% mannose (ND+M), (3) 45% HFD, and (4) 45% HFD with 2% mannose (HFD+M). HFD mice gained much more weight over time compared to ND mice. Surprisingly, body weights of HFD+M mice were similar to ND group (Figure 1A). HFD-fed female C57BL/6J mice supplemented with 2% mannose were also resistant to weight gain (Figure S1). Because we observed similar effects in both male and female mice, we performed subsequent experiments using male C57BL/6J mice. Substituting 2% galactose for mannose in drinking water did not attenuate HFD-induced weight gain (Figure 1B), indicating a mannose-specific effect. The effects were similar when mannose was supplied at 3 weeks post-weaning (PW) (Figure 1C); however, the differences between HFD and HFD+M mice were less pronounced than when mannose was started at weaning (Figure 1A). We next determined whether mannose was required continuously throughout the intervention to yield benefits. We weaned mice on HFD+M and maintained it for 13 weeks. At this time, mannose was removed from the drinking water, causing increased weight gain within 3 weeks of removal (Figure 1D). Weight gain attenuation was also observed when ND mice were supplemented with mannose but less pronounced, suggesting that mannose has a greater effect in the context of HFD (Figure 1E). Therefore, subsequent experiments were focused on HFD.

HFD+M mice had reduced fat mass as compared to HFD mice at 13 weeks (Figure 1F). HFD-fed mice provided mannose 3 weeks PW also showed significant reduction (~30%) in fat mass compared to unsupplemented HFD mice (Figure 1G). The reduction in fat mass was evident by the third week of supplementation and reached statistical significance by the fifth week (Figure 1G). Mannose had similar effects on mice fed a 60% HFD (Figure 1H). Histological analysis of adipocytes (150 cells/mouse) from epididymal fat ($n = 4$) showed significantly smaller adipocytes in HFD+M mice compared to HFD (ND: $2,086 \pm 96 \mu\text{m}^2$; HFD: $5,501 \pm 547.2 \mu\text{m}^2$; HFD+M: $2,271 \pm 336 \mu\text{m}^2$; $p < 0.005$) as shown in Figure 1I (top). HFD+M mice (45% or 60%) had reduced liver steatosis compared to those fed HFD alone (Figure 1I, middle and bottom). Because observations were similar with 45% and 60% diet, subsequent experiments used 45% HFD.

Mannose Improves Glucose and Lipid Homeostasis and Reduces Gene Expression of Inflammatory Markers in Adipocytes of HFD Mice

Glucose and insulin levels were higher in HFD mice compared to ND but normalized when mannose was initiated at weaning (Table 1). HFD+M mice had significantly elevated total cholesterol primarily due to a marked increase in high-density lipoprotein (HDL), whereas low-density lipoprotein (LDL) or very-low-density lipoprotein (VLDL) was unaffected.

Lower serum glycerol in HFD+M mice indicates reduced triglyceride hydrolysis. Additional blood parameters examined were not significantly altered in HFD+M compared to HFD mice (Table 1).

Reduced glucose tolerance and insulin sensitivity are associated with HFD. After 16 weeks, HFD mice were glucose intolerant compared to ND mice (Figures 1J and 1K). Mannose supplementation improved glucose tolerance, as shown by lower area under the curve (Figure 1K). HFD did not induce insulin insensitivity (Figures 1L and 1M). However, HFD +M mice displayed a slight increase in insulin sensitivity, as shown by a greater slope of glucose fall in HFD+M compared to both ND and HFD mice (Figure 1M).

Microarray gene expression analysis showed 1,142 differentially expressed genes (>1.5-fold) in epididymal fat (n = 3) of HFD+M and HFD, among which 756 genes were also differentially expressed in HFD versus ND. Overall, the gene expression patterns of HFD+M mice more closely resembled that of the ND group. Inflammatory pathways and predicted upstream regulators, such as tumor necrosis factor alpha (TNF- α) and interferon γ (IFN γ), were inhibited in HFD+M mice (Table S1), which were activated in HFD mice.

Mannose Increases Aerobic Endurance and Physical Fitness in HFD Mice

Mannose feeding had no effect on food and water intake, and X and Z activity, O₂ consumption, CO₂ production, and energy expenditure were unaltered (Figure S2). Leptin levels, which increase satiety, were also unaltered (Table 1). Therefore, mannose-induced reduction in weight and fat mass is neither due to reduced food intake or increased activity or energy expenditure in HFD+M mice. Importantly, HFD+M mice displayed increased maximal oxygen consumption (VO_{2max}) (HFD+M: 822.6 $\pm$ 176.2 mL/kg/hr; HFD: 285.4 $\pm$ 153.6 mL/kg/hr; n = 5; p = 0.05), achieved higher speed (HFD+M: 25 $\pm$ 0.8 m/min; HFD: 22.8 $\pm$ 0.8 m/min; n = 5; p = 0.06), and stayed on a treadmill for longer duration (HFD+M: 13.0 $\pm$ 0.3 min; HFD: 10.8 $\pm$ 0.6 min; n = 5; p = 0.01) relative to HFD mice, indicating increased physical fitness with mannose.

Metabolic Analysis of Mannose-Supplemented HFD Mice

We analyzed host metabolism of HFD and HFD+M mice to explain the beneficial effects of mannose. Additional plausible metabolic factors could be (1) lower triglyceride synthesis and rapid VLDL clearance, (2) increased gluconeogenesis, (3) AMP kinase (AMPK) activation, and (4) increased fatty acid oxidation (FAO). We assessed all these factors. Mannose did not alter the rate of triglyceride synthesis (HFD: 1,880 $\pm$ 116.2 mg/dL/hr; HFD +M: 1,957 $\pm$ 144.5 mg/dL/hr; n = 8) or VLDL clearance (HFD: 72.29 $\pm$ 13.29 ng/mL/min; HFD+M: 86.43 $\pm$ 12.02 ng/mL/min; n = 7). AMPK activation is important for nutrient sensing, cellular glucose uptake, and energy homeostasis (Carling, 2017; Hardie et al., 2012). We measured the phosphorylated form, AMPK α [pT172], in mouse liver and muscle tissue. Mannose did not significantly change AMPK α [pT172] levels in liver (data not shown). However, in muscle, HFD increased phosphorylated AMPK α (27.29 $\pm$ 6.2 units/mg; n = 8; p = 0.03) compared to ND (11.78 $\pm$ 1.24 units/mg; n = 8), which was not observed in HFD+M mice, and the levels were comparable to ND (HFD+M: 13.11 $\pm$ 1.5

units/mg; $n = 8$). Adiponectin, which plays a significant role in insulin sensitivity (Lihn et al., 2005), was similar in HFD and HFD+M mice (Table 1).

We used qPCR to determine expression changes of metabolic enzymes in the liver. Although the expression of phosphomannomutase that diverts mannose-6-phosphate to glycosylation was unaltered by mannose, MPI that catabolizes mannose through glycolysis was 1.25-fold higher in HFD+M versus HFD group ($n = 8$; $p = 0.009$). Other glycolytic enzymes, like glucokinase, phosphofructokinase, and pyruvate kinase, did not differ in expression in HFD versus HFD+M (data not shown). Mannose did not affect key gluconeogenesis enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Higher expression of peroxisome-proliferator-activated receptor alpha (*PPAR α*), carnitine palmitoyltransferase 1 (*Cpt-1*), and peroxisomal membrane protein (*Pex-11a*) with mannose may suggest the possibility of increased FAO in HFD mice. Although the expression of these genes was increased in HFD ($n = 8$) compared to ND (*PPAR α* : 2.0-fold, $p < 0.0001$; *Cpt-1*: 5.3-fold, $p = 0.02$; *Pex11a*: 2.5-fold, $p < 0.0001$), such increases were not observed in HFD+M mice and were comparable to ND mice, which is consistent with lower lipid content in HFD+M liver. These results ruled out direct effect of mannose on any of these host metabolic pathways.

Mannose-Fed HFD Mice Have Higher Fecal Energy Content, Mannose, and SCFAs

We determined energy utilization by measuring fecal energy content (Table 2). Feces from HFD+M mice had ~ 1.85 Kj/g or 442 cal/g more energy content compared to HFD, indicating decreased absorption of dietary energy with mannose. This interpretation is reinforced by the energy content of ND mice (16.06 ± 0.12 Kj/g; $n = 14$) that was similar to HFD mice (16.09 ± 0.17 Kj/g; $n = 14$; $p = 0.01$). Higher fecal energy content in HFD+M mice could be due to increased mannose. HFD+M mice had 40-fold more fecal mannose compared to HFD, whereas glucose levels remained unchanged (Table 2). However, higher mannose contributed less than 1% (0.0176 Kj/g) of the total elevated energy content (~ 1.85 Kj/g). Mannose increased total fecal short-chain fatty acid (SCFA) concentration, including acetate, propionate, and, most notably, butyrate (Table 2), in HFD+M compared to HFD. However, the increase in fecal SCFA accounted for less than 0.5% of the observed energy content difference (acetate: 0.003 Kj/g; propionate: 0.0004 Kj/g; butyrate: 0.0006 Kj/g). These results indicate as yet unidentified dietary components not absorbed in mannose-treated mice, which is consistent with their lean phenotype.

Beneficial Effects of Mannose Are Lost when Started Later in Life

We next assessed the utility of mannose treatment as an intervention after onset of diet-induced obesity (DIO). We weaned 16 mice on HFD. 8 mice were supplemented with 2% mannose at 8 weeks PW. Mice gained weight (42.9 ± 1.4 g; 19 weeks) and fat mass (13.5 ± 0.9 g) on HFD as expected. Surprisingly, mannose neither reduced weight (43.1 ± 0.8 g; 19 weeks) or fat mass (13.6 ± 0.8 g), implying that mannose-mediated protection from DIO is lost unless mannose is provided early in life. This, coupled with the fact that continuous mannose supplementation is required, suggests that mannose exerts its effects via a factor that can be modulated early in life and persists only as long as mannose is provided.

Mannose Alters Gut Microbial Composition of HFD Mice

We speculated that the alterations occurring during milk to solid food diet transition might be associated with the loss of mannose-induced lean phenotype when administered later in life. We compared the fecal microbiota composition of mice weaned on ND, HFD, and HFD +M, where mannose was initiated at 0, 3, and 8 weeks PW. Fecal samples were analyzed after 8–10 weeks at ages 13, 14, and 19 weeks, respectively (Figure 2A).

Obese HFD mice had reduced Bacteroidetes and increased Firmicutes and Actinobacteria compared to lean ND mice (Figure 2B). Mannose increased Bacteroidetes:Firmicutes ratio 2.3- and 1.5-fold when started at 0 and 3 weeks PW, respectively, whereas it was unaltered (1.1-fold) when initiated at 8 weeks PW, establishing an association between microbiota composition (lean versus obese) and the timing of mannose introduction. At the genus level, *Bacteroides* spp. were relatively abundant in mannose responsive mice and increased when mannose was started at 0 weeks (HFD: $13\% \pm 3.2\%$; HFD + M: $23\% \pm 1.6\%$; $p = 0.02$) or at 3 weeks (HFD: $18\% \pm 2.5\%$; HFD+M: $26.2\% \pm 3.3\%$; $p = 0.03$); however, its relative abundance decreased precipitously in mice when mannose was started at 8 weeks PW (HFD: $0.001\% \pm 0.0002\%$; HFD $\pm$ M: $0.0005\% \pm 0.0001\%$; $p = 0.0006$). A reciprocal signature was observed for *Faecalibacterium* spp. and *Clostridium* spp. that display higher relative abundance in 8 weeks PW mice compared to 0 weeks and 3 weeks PW mice (Figure 2C). These signatures were also reflected at the species level (Figure 2D).

To further solidify the association of mannose-induced alterations in microbiota composition and adiposity, we assessed microbial communities in HFD+M mice following removal of mannose supplementation that resulted in compositional changes resembling that of the HFD microbiota (Figure S3). These changes tracked with weight gain observed upon mannose removal (Figure 1D) and highlight that the effects of mannose when introduced at weaning are durable but are required continuously to retain microbiota composition and the lean phenotype.

Mannose Reduces Energy Harvest by Gut Microbes in HFD Mice

Our results implicate the gut microbiota as a potential factor mediating mannose-induced lean phenotype. We did transcriptomics of cecal contents from mice on HFD and HFD+M to gain mechanistic insights. To minimize the effects associated with transition from milk to solid food diet, we compared the transcriptome of cecal microbiota where mannose was introduced at 3 weeks PW (mannose responsive) to when mannose was initiated at 8 weeks PW (mannose non-responsive). We focused primarily on differential transcript abundance, comparing HFD and HFD+M for both sets at the level of taxonomy and function.

Taxonomy—We examined the distribution of contigs mapped to reference genomes to identify taxa with differential transcript abundance following mannose treatment (Table S2). We applied a cutoff of 3-fold change and $p < 10^{-5}$ (Kal's Z test) for differential abundance. Most differentially abundant transcripts mapped to Firmicutes (Figure 3A; Table S3), nearly 50% of which mapped to Lachnospiraceae in both mannose responsive and nonresponsive mice (Figure 3B). Notably, 3,853 contigs expressed by *Bacteroidetes* (~60% from *Bacteroides* spp.) were differentially abundant (HFD versus HFD+M) in mannose-

responsive mice compared to only 115 contigs in non-responsive mice (Figure 3A; Table S3). This fits with 16S rDNA analysis (Figure 2A), although the magnitude of differential transcript abundance exceeded the change in estimated cellular abundance within the community. The majority of mannose-induced differential transcript abundance mapped to 17 genera (Figure 3B; Table S4).

Functional Analysis—Mannose treatment of HFD mice initiated at 3 weeks and 8 weeks PW altered multiple cellular processes (Figure 3C; Table S5). There were 2,328 mannose-induced differentially abundant transcripts expressed by *Bacteroides* and *Parabacteroides* in responsive mice compared to only 46 contigs in non-responsive mice (Table S6). This is significant because many *Bacteroides* species encode carbohydrate utilization machinery (*sus* genes) comprised of more than 100 glycosyl hydrolases (GHs) and starch binding proteins, enabling glycan catabolism of a variety of dietary polysaccharides (Wexler, 2007). Interestingly, although the relative abundance of *Bacteroides* spp. based on 16S rDNA increase from 18% to 28% at 3 weeks PW, mannose induces a global transcription repression in these glycan-degrading genera (Table S6). We hypothesized that mannose may act as a strong transcription regulator, impacting energy harvest (microbial glycan hydrolysis, sugar metabolism, and transport pathways). Indeed, ~10% of all mannose-induced differentially abundant transcripts mapped to carbohydrate-related pathways.

Glycan Hydrolysis—Gut microbial GHs are important for energy harvest. 2% or 3% of the differentially expressed contigs mapped to GH genes. We observed notable differences between responsive mice and non-responsive mice (Figure 3D; Table S7). In responsive mice, 42% of GH transcripts mapped to *Firmicutes* and 57% to *Bacteroidetes*, out of which 50% mapped to *Bacteroides* spp. and were reduced by 3.8-fold by mannose. In non-responsive mice, differentially abundant GHs were distributed predominantly among *Firmicutes*, particularly, *Clostridium* spp. Nearly 15% of GH transcripts encoded β -galactosidase, which were reduced in abundance to a greater extent in mannose-responsive mice (2.8-fold) compared to non-responsive (1.3-fold). A similar trend was observed for GH family 25, GH family 109, and chitinase. Although less abundant, overall sialidase expression was reduced by 3-fold in responsive mice but increased by 3-fold in non-responsive mice (Figure 3D). Sialic acid production is linked to pathogen proliferation and intestinal inflammation (Huang et al., 2015). Mannose also reduced the transcript abundance of α -1,6-mannanase by 24-fold and α -1,2-mannosidase by 2.5-fold in mannose-responsive mice (Figure 3D). Our results suggest that the presence of excess mannose in the gut downregulates GH activity in mannose-responsive mice but less so in non-responsive mice.

Carbohydrate Metabolism—Consistent with reduced GH activity, mannose reduced overall transcript abundance related to carbohydrate metabolism in mannose-responsive mice compared to non-responsive mice (Table S8) reflected by transcripts encoding glycolysis and/or gluconeogenesis, pyruvate metabolism, glycogen biosynthesis, and catabolism (Figure 3E). There were 80-fold more differentially expressed transcripts encoding functions related to mannose metabolism in mannose-responsive mice as compared to non-responsive mice, which increased by 3.6fold with mannose. In non-

responsive mice, glycolysis and/or gluconeogenesis, pyruvate metabolism, glycogen catabolism, and starch sugar and amino sugar metabolism increased (Figure 3E; Table S8).

Transcriptomics analysis suggests that the beneficial effects of mannose in responsive mice are in part due to reduced energy harvest by gut microbes, especially *Bacteroides* spp., because of the reduced expression of GHs that are expected to result in reduced energy (calorie) provision for the host (Figure 3F). Reduced glycan catabolism is reflected in reductions in downstream sugar metabolism pathways.

DISCUSSION

Dietary sugars, like fructose and sucrose, are associated with poor health and childhood obesity (Goran et al., 2013). Here, we show that another simple sugar, mannose, prevents HFD-induced obesity in a C57BL/6J mouse model. This is the first report of mannose influencing host energy metabolism. Surprisingly, mannose provided in early life abrogated adverse effects of HFD consumption. HDL levels were significantly elevated in HFD+M versus HFD mice. HDL is associated with transport of cholesterol and fatty acids out of arterial walls, thus reducing inflammation and preventing atherosclerosis (Feig et al., 2014). Food intake and activity in both groups were similar; however, mannose increased fecal energy content (by 1.85 Kj/g or 0.442 Kcal/g) in HFD+M compared to HFD mice, suggesting that mannose impacts dietary nutrient absorption, thereby reducing adiposity in mannose-treated mice.

A particularly interesting aspect of our results is the temporal effect of mannose on host metabolism. Mannose prevents HFD-induced obesity only when initiated early in life, but not when provided later. This suggested the gut microbiome as a plausible factor, which is in a dynamic state during early life. Profiling HFD and HFD+M microbiota revealed robust associations between obese phenotype and altered Bacteroidetes:Firmicutes ratio. This is a common signature seen in both diet-induced and genetically obese mouse models and humans (Ley et al., 2005, 2006; Lin et al., 2012; Ridaura et al., 2013), although some of the other studies do not corroborate this association. This signature matched with progressive loss of the mannose-induced lean phenotype, depending on the time at which mannose was started, and becomes mannose insensitive as the gut microbiota adapts to a solid food diet. The mannose dependency of this signature is reinforced by the mannose removal experiment that led to microbiota convergence to the HFD signature. The loss of mannose responsiveness could also be due to the age- and diet-dependent changes in host metabolism. The sugar composition of intestinal and colonic mucin undergoes a shift corresponding temporally to the shift to a solid food diet. Fucosyl transferase activity is reduced and sialic acid transferase are increased (Rodríguez et al., 2015), which may have a strong impact on the relative fitness of mucosa-associated gut microbes that derive much of their energy from mucin foraging.

Cecal transcriptomics demonstrated that mannose strongly influenced the functional expression of gut bacterial communities in HFD mice. Reduced energy harvest correlates with reduced transcript abundance of genes encoding GH, leading to reduced expression of carbohydrate metabolism pathways in 3 weeks PW mice, but not in 8 weeks PW mice.

Decreased bacterial energy harvest may partially account for mannose-mediated lean phenotype. This result is similar to an earlier metagenomic study of genetically obese mice (ob/ob) demonstrating increased abundance of GHs to be associated with greater energy harvest and obesity, resulting in reduced fecal energy content (by ~0.2 Kcal/g; Turnbaugh et al., 2006). In our study, transcripts related to butyrate and propionate production with mannose were unchanged. Therefore, increased abundance of fecal SCFAs in HFD+M mice may be due to reduced host absorption. The increased fecal energy content and SCFAs strongly suggests a mannose-dependent influence on energy absorption in the gut. The potential involvement of intestinal microbiota mediating altered lipid absorption is also a possibility that is supported by a recent report demonstrating that small intestinal microbiota regulate lipid absorption in a HFD-specific manner (Martinez-Guryn et al., 2018).

Mannose caused a sharp decrease in differentially expressed genes among Bacteroidetes, a signature driven strongly by *Bacteroides* spp. This shift has significant effects on GH and sugar metabolism in these taxonomic groups. Species such as *B. acidifaciens* and *B. uniformis* ameliorate obesity by immunomodulation and improved host metabolism (Gauffin Cano et al., 2012; Yang et al., 2017). However, the mechanism for these effects is unknown.

We considered three approaches to evaluate the causative effect of gut microbiota in the observed phenotypes: (1) antibiotic ablation of gut microbiota would blunt the lean phenotype. The well-known impact of antibiotics on host energy balance pathways (Cho et al., 2012; Cox et al., 2014), independent of mannose driven effects, would not allow definitive conclusions; (2) Provide HFD and HFD+M diets to GF mice to evaluate mannose-mediated lean phenotype in a microbiota-independent manner. However, GF mice are resistant to HFD weight gain (Bäckhed et al., 2007), thereby negating our ability to assess the effects of mannose; and (3) colonize GF mice with HFD+M microbiota from responsive mice to determine whether it could induce lean phenotype. However, our results indicate that a lean phenotype requires continuous mannose supplementation. Its cessation reconfigures microbiota to an obese signature, precluding expectations that HFD+M microbiota would confer beneficial effects. Despite, the limitations inherent to establishing causality in the mannose lean phenotype model, alternative approaches may be possible, including co-colonization of GF mice with defined consortia that permit HFD weight gain with candidate species that may mediate the effects. An alternative to a probiotic approach is a prebiotic approach to maintain lean microbiota signatures and phenotype. Finally, metabolomics analysis comparing mannose-induced metabolites generated by mannose responsive mice may allow the testing of these candidate molecules on host energy balance pathways.

Although we have extensively characterized host metabolic pathways involved in sugar and fat metabolism, our data do not rule out the possibility of additional direct effects of mannose on the host. Mannose can be taken up by all host cells (Sharma and Freeze, 2011) but may have a cell-type-specific effect that influences fat absorption and/or metabolism either directly or indirectly. A recent study has shown mannose supplementation of non-obese diabetic mice prevents onset of diabetes by increasing the number of T_{reg} cells (Zhang et al., 2017).

In summary, we show that mannose supplementation early in life confers resistance to weight gain, liver steatosis, and glucose intolerance caused by HFD, which could be partially explained by higher fecal energy content and changes in gut microbiome. This study highlights that simple sugars like mannose that are not typical host energy sources can have profound effects on host metabolism, gut microbiota, and energy harvest from the diet.

EXPERIMENTAL PROCEDURES

Animals

Institutional Animal Care and Use Committee of Sanford-Burnham-Prebys Medical Discovery Institute (SBP-Discovery) approved all the animal studies. We purchased C57BL/6 mice from Jackson Laboratories. HFD (TD.06415) and ND (ND 2018) were bought from Harlan Laboratories. All mice were maintained on a 12 hr dark/12 hr light cycle. For the experiments, we bought 24–32 male C57BL/6J mice (19 days old) directly from Jackson Laboratories. Mice were randomized and weaned on either ND or HFD or HFD and 2% mannose in drinking water and fed *ad libitum*. In some experiments, mannose was supplemented 3 weeks or 8 weeks PW. All mice were housed in the same room to minimize any environmental effects.

Materials

Chemicals and reagents were ordered from Sigma-Aldrich unless specified otherwise. D-mannose was purchased from Hofman International. Blood insulin ELISA kit was bought from ALPCO immunoassays (cat no. 80-INSMS-E01). Adiponectin and leptin kits were procured from BioVendor Research and Diagnostic products. Assay reagent kits from BioAssay Systems were used for measuring total cholesterol, LDL/VLDL, HDL (EHDL-100), triglycerides (ETGA-200), free fatty acids (EFFA-100), and glycerol (EGLY-200). RNeasy Plus Universal and RNeasy Lipid tissue kits for RNA isolation from mouse tissues and QiaAmp Fast DNA Stool Mini kit for fecal DNA isolation were purchased from QIAGEN, Hilden, Germany. Invitrogen Superscript II reverse transcriptase and Applied Biosystems PowerSYBR Green PCR master mix (Thermo-Fisher Scientific) were used for qPCRs.

Software

Ingenuity pathway analysis (IPA) software (QIAGEN, Hilden, Germany) was used to analyze mouse microarray data (<https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/>). CLC genomics workbench and CLC microbial workbench from QIAGEN was used for sequencing analysis (<https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/>). Annotations were performed using Blast2go plug-in within CLC workbench (<https://www.blast2go.com/>).

Body Weight and Fat Mass Measurement

Body weights were monitored weekly starting at weaning. Fat mass was determined every 2 or 3 weeks in conscious mice using LF90II TD-NMR instrument (Bruker) over a period of 3 or 4 months. In some experiments, fat measurements were performed at the time of sacrifice (16 weeks) by collecting and weighing various fat pads.

Histology

Mouse fat pads were fixed in Z-fix (Anatech) at room temperature for 24 hr followed by an additional 24 hr at 4°C, washed four times with PBS, and embedded in paraffin. 5-µm-thick sections were cut and stained with H&E for structural analysis using Aperio's scanscope slide scanning system (Aperio Technologies). We did morphometric measurements with Aperio Scanscope software, Imagescope. For oil red O staining, the livers were fixed and washed as described above followed by transfer to 30% sucrose and embedded in OCT cryo-embedding compound. Frozen sections were cut and stained with oil red O.

Blood Chemistry

Glucose levels were determined in the blood drawn from fasted mice (5 hr) by using FreeStyle glucometer and glucose strips (Abbot). Parameters of freshly drawn blood were measured by using VetScan VS2 instrument (Abaxis). Blood insulin, adiponectin, and leptin levels were measured using ELISA kits. Total cholesterol, LDL/VLDL, HDL, triglycerides, free fatty acids, and glycerol were measured using assay reagent kits. Details of the kits are mentioned elsewhere in this section.

Metabolic Analysis

Energy balance measurements were made using a Comprehensive Lab Animal Monitoring System (Columbus Instruments). Mice were acclimated to the Comprehensive Lab Animal Monitoring System (CLAMS) for at least 48 hr prior to acquisition of data. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured every 15 min for calculation of the respiratory exchange ratio ($\text{RER} = \text{VCO}_2/\text{VO}_2$) and energy expenditure ($\text{EE} = 3.815 + [(1.232 \pm 3 \text{ RER}) \pm 3 \text{ VO}_2]$ in $\text{kCal}/[\text{kg} \pm 3 \text{ hr}]$). Food and water intake were measured every 15 min using a precision scale and volumetric drinking monitor, respectively. Ambulatory activity was estimated by the number of infrared beams broken along the x axis of the metabolic cage.

$\text{VO}_{2,\text{max}}$ Test

VO_2 during exercise was measured in mice placed in an enclosed treadmill connected to the CLAMS (Columbus Instruments). Mice were allowed to acclimate on the treadmill for 30 min. Resting VO_2 was determined as the average of measurements over the last 10 min of the acclimation period. Mice then began running at 10 m/min, 0% grade, and the speed was increased by 4 m/min every 3 min until the mice reached exhaustion. Mice were encouraged to run with the use of an electric grid placed in the back of the treadmill (1.5 mA; 200-ms pulses; 4 Hz). Mice were defined as exhausted when they spent more than 5 continuous seconds on the electric grid. $\text{VO}_{2,\text{max}}$ was achieved when VO_2 no longer increased despite an increase in treadmill speed.

Glucose Tolerance Test and Insulin Tolerance Test

For glucose tolerance tests (GTTs), mice were fasted for 5 hr. A baseline blood glucose reading was obtained from the tail at the end of the fast. Mice were injected with glucose (1.5 g/kg, intraperitoneally), and blood glucose was measured at 0, 15, 30, 60, and 90 min. Area under the curve (AUC) above baseline was calculated as an index of glucose tolerance.

For insulin tolerance tests (ITTs), mice were fasted for 5 hr. Following a baseline glucose reading, mice were injected intraperitoneally with insulin (0.75 U/kg) and blood glucose levels were measured at 0, 15, 30, 60, and 90 min. The fall in the slope of the glucose curve from 0 to 30 min was used as an index of insulin sensitivity.

Measurement of Fecal Energy Content

1.0 g of feces was dried, powdered, and subjected to analysis in triplicates using Parr 1266 Bomb calorimeter with water-handling and temperature-control systems. Energy value per mass dry weight was then calculated.

Microarray Expression Analysis

Labeled cRNA was prepared from 500 ng RNA using the Illumina RNA Amplification Kit from Ambion (Thermo-Fisher Scientific). 1,500 ng labeled cRNA was hybridized overnight at 58°C to the Sentrix MouseWG-6 Expression BeadChip (>46,000 gene transcripts; Illumina) according to the manufacturer's instructions. BeadChips were subsequently washed and developed with fluorolink streptavidin-Cy3 (GE Healthcare). BeadChips were scanned with an Illumina BeadArray Reader.

Expression Analysis by qPCR

Genomic DNA was prepared from freshly frozen mouse tissues. Most primers are from PrimerBank (Spandidos et al., 2010) with mentioned ID. List is as follows:

Phosphomannose isomerase: ID 142345292c1

Forward 5'-CCGCGAGTGTTCCTCCACTTT-3'

Reverse 5'-GTCCGGGTTTTCAGCAATCCA-3'

Phosphomannomutase2: ID 133892745c1

Forward 5'-TCTGTCTCTTCGACATGGATGG-3'

Reverse 5'-CCACTCCAATTTTGGTCTTCTGC-3'

Glucokinase: (Dentin et al., 2004)

Forward 5'-CCCTGAGTGGCTTACAGTTC-3'

Reverse 5'-ACGGATGTGAGTGTTGAAGC-3'

Pyruvate kinase: (Dentin et al., 2004)

Forward 5'-CTTGCTCTACCGTGAGCCTC-3'

Reverse 5'-ACCACAATCACCAGATCACC-3'

Phosphofructokinase: ID 31560653a1

Forward 5'-GGAGGCGAGAACATCAAGCC-3'

Reverse 5'-CGGCCTTCCCTCGTAGTGA-3'

Phosphoenolpyruvate carboxykinase: ID 142382732c2

Forward 5'-AAAGCAAGACGGTGATTGTAAC-3'

Reverse 5'-GCACTCCAATGCGGGAGAG-3'

Glucose-6-phosphatase: ID 47271523b2

Forward 5'-CACCTTTCTGGGCGATCCTAA-3'

Reverse 5'-GTACCCGGATTCATGCACCC-3'

Peroxisome proliferator-activated receptor α :

Forward 5'-GGGCTCTCCCACATCCTT-3'

Reverse 5'-CCCATTTCGGTAGCAGGTAGTC-3'

Carnitine palmitoyltransferase 1: (Cabrero et al., 2002)

Forward 5'-TTCAGTGTGACCCAGACGG-3'

Reverse 5'-AATGGACCAGCCCCATGGAGA-3'

Peroxisomal membrane protein Pex11: (Hall et al., 2010)

Forward 5'-CACTGGCCGTAAATGGTTCA-3'

Reverse 5'-GCTTGGATGCTCTGCTCAGTT-3'

Preparation of Fecal Extract for Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

1.0 mL of 1:1 mixture of methanol and water (pH adjusted to 10.5 by NaOH) containing 50 nmols 2-ethyl butyric acid (2-EBA) and 5 nmols arabitol was added to 0.1 g of ground feces. The fecal suspension was vortexed repeatedly over a course of 30 min followed by centrifugation at 3,000 rpm for 10 min to remove coarse particles. The supernatant was centrifuged again at 12,000 rpm for 10 min. The supernatant (fecal extract) was collected and stored at 20°C.

SCFA Analysis by GC-MS

The standards were prepared from the Supelco Volatile Acid Standards Mix (Sigma; 46975-U), mixed with an equal concentration of 2-EBA and pH adjusted to 9.5, and O-ethylhydroxylamine (20 mg/mL in pyridine; 30 μ L) was added to dried fecal extracts (50 μ L) or varying amounts of dried standards and incubated at 80°C for 20 min, followed by addition of 30 μ L MTBSTFA (Soltec) and incubation at 80°C for an hour. The samples were cooled and analyzed by gas chromatography-mass spectrometry (GC-MS) analysis on a Shimadzu QP2010 Plus GC-MS with 0.5 mL injection. GC was programmed with an injection temperature of 250°C, split ratio 1/10; GC oven temperature was initially 50°C for 4 min, rising to 124°C at 6°C/min, and to 280°C at 50°C/min with a final hold at this temperature for 2 min. GC flow rate with helium carrier gas was 50 cm/s through a 15 m $\times$ 0.25 mm $\times$ 0.25 mm SHRXI-5ms (Shimadzu) column. GC-MS interface temperature was 300°C (electron impact), and MS ion source temperature was 200°C, with 70 eV ionization voltage. SCFAs in samples were quantified using calibration curves based on peak areas of SCFAs in standards.

Mannose Estimation in Feces by GC-MS

50 µL fecal extracts, mannose standards, and arabitol (internal standard) were dried. 10 µL water and 50 µL hydroxylamine hydrochloride in 1-methyl-imidazole were added to the samples and standards. The samples were heated at 65°C for 30 min followed by cooling. Acetic anhydride (100 µL) was added and heated at 65°C for 30 min. After cooling, the derivatized sample was extracted with chloroform and water a couple of times. Chloroform layer was collected and dried. The samples were then analyzed by GC-MS as described earlier (Ichikawa et al., 2014).

Microbiota Analysis by 16S Sequencing

Genomic DNA was isolated from mouse feces using QiaAmp DNA stool kit (QIAGEN), with an additional step of bead beating for 5 min with 0.1 mm beads to ensure maximum lysis of bacterial cells. Multiplexed libraries were prepared according to the protocol from Illumina using V3-V4 region of 16S rDNA and HiFi HotStart DNA Polymerase (Kapa Biosystems) for amplification. Final amplified products were quantified by ABI Prism library quantitation kit (Kapa Biosystems). Each sample was diluted to 10 nM, and equal volume from each sample was pooled. The quality of the library was checked by Bio-Rad Experion bioanalyzer (Bio-Rad). Illumina MiSeq platform was used for sequencing. We performed paired end reads (250 bases) and multiplexed 96 samples/lane in order to generate ~200,000 sequences/sample. Subsequent analysis was done using QIAGEN's CLC Microbial genomics module 2.5. Paired end reads were merged (mismatch cost: 2; minimum score: 8; gap cost: 3; maximum unaligned end mismatches: 0) and trimmed to same length. Additional quality filter steps were applied to exclude the chimeras and to ensure comparable high coverage in all samples with at least 50,000 reads. A multi-step algorithm clustered the reads to operational taxonomy units (OTUs) or phylotypes based on 97% identity. We used Greengenes database (v13_5 99%) for taxonomic classification of sequences and assigned each to particular clusters. Each unique OTU was subjected to BLAST against NCBI 16S database to identify closest match to the taxa at genus and species level based on lowest e-value and identity %.

RNA-Seq Library Preparation

Cecal contents from mice (n = 8) were collected, immediately frozen, and stored at 80°C until further processing. RNA from each mouse for each diet group was isolated individually using Ambion RiboPure Bacteria kit (Thermo Fisher Scientific). 1 µg cecal RNA each from 8 mice/diet group was pooled to generate 1 pool/diet for library preparation. The quality of total RNA was assessed by the Agilent Bioanalyzer Nano chip (Agilent Technologies). Approximately 2 µg of total RNA was Ribo-depleted using Ribo-Zero Gold rRNA kit (Epidemiology) from Illumina. RNA-seq libraries were constructed from the recovered non-ribosomal RNAs using Truseq Stranded total RNA library preparation kit (Illumina) as per the instructions. RNA was fragmented into small pieces using divalent cations under elevated temperature. The first strand cDNA was synthesized using random primers followed by second strand synthesis using DNA Polymerase I. cDNA was then ligated with index adapters for each sample followed by purification and amplification by PCR to create the final library. The quality and quantity of the library was established using an Agilent

Bioanalyzer and Kapa Biosystems qPCR. Multiplexed libraries were pooled and single-end 50-bp sequencing was performed on one flow cell of an Illumina Hiseq 1500.

RNA-Seq Data Analysis

RNA-seq analysis was performed on equal mass RNA pools derived from cecal material to monitor expression of genes and pathways of interest. We employed QIAGEN's CLC workbench 10.0 to identify differentially expressed genes and functional groups. 200 million reads from all the samples were combined to generate a reference, and *de novo* assembly was done using an algorithm based on de Bruijn graphs to obtain the contigs (114,502 for 3 weeks PW and 153,974 for 8 weeks PW group of mice; Table S2). Reads from individual samples (HFD and HFD+M) were mapped to these contigs. Fold change HFD+M versus HFD was obtained for each contig. In parallel, we performed BLASTx searches (e value < 10^{-5}) of all contigs against a subset of bacterial RefSeq protein database containing bacterial reference genomes, featuring mainly the members of gut microbiota that provided NCBI identifiers, predicted protein function, and approximate taxonomic source of transcripts to each contig. The contigs were further annotated using Blast2go annotation software that provided gene ontology numbers. For differential transcript abundance (HFD versus HFD+M), we applied a significance cut off of $p < 10^{-5}$ (Kal's Z test for single determinations) and fold change of 3 to individual contigs in compared groups, leaving 14,569 contigs (3 weeks PW) and 11,197 contigs (8 weeks PW). Further annotation of this set was done manually using databases (Kyoto Encyclopedia of Genes and Genomes [KEGG] and Uniprot) and literature. RPKM values were then aggregated to determine differential transcript abundance for particular taxa or functional role. Fold change between HFD+M and HFD was calculated as shown in Tables S2, S3, S4, S5, S6, S7, and S8. The heatmaps for the most abundant and relevant metabolic pathways or function were prepared using R studio (RStudio, Boston, MA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Providing mannose in early life prevents high-fat-diet-induced obesity in mice
- Mannose does not benefit when initiated post-obesity onset (8 weeks post-weaning)
- Mannose-induced lean and fit phenotype correlates with gut microbiota changes
- Less energy harvest by microbiota partly explains mannose-mediated lean phenotype

Figure 1. Mannose Induces a Lean Phenotype, Reduces Liver Steatosis, and Improves Glucose Tolerance

(A–E) Body weights were measured weekly for C57BL/6J mice weaned at the age of 3 weeks on either ND or 45% HFD without or with (A) 2% mannose at weaning, (B) 2% galactose at weaning, (C) 2% mannose started 3 weeks PW, (D) 2% mannose started at weaning and continued for 16 weeks followed by its removal, and (E) 2% mannose at weaning.

(F–H) Fat mass measurements. Total fat mass is the sum of all fat pads collected at age 16 weeks (F). Mice weaned on 45% HFD, and mannose started at 3 weeks PW (G). Fat mass of mice weaned on 60% HFD and 2% mannose (H).

(I) Histology images are representative of analysis from 4 mice in each diet group. Top: H&E staining of epididymal adipocytes is shown. Middle and bottom: oil red O staining of frozen liver sections from mice is shown.

(J–M) Three-week-old C57BL6/J mice were weaned on ND or HFD or HFD+2% mannose.

(J and K) Glucose tolerance test: fasted mice were injected with glucose (1.5 g/kg), and blood glucose was measured at 0, 15, 30, 60, and 90 min. Glucose clearance (J). Area under the curve indicates glucose levels (K).

(L and M) Insulin tolerance test: mice were injected with insulin (0.5 U/kg), and blood glucose levels were measured at 0, 15, 30, 60, and 90 min. Glucose clearance (L). The fall in the slope of the curve is the measure of insulin sensitivity (M).

The data presented in the graphs are the mean $\pm$ SEM of indicated number of mice (N).

Significance was calculated for HFD+M versus HFD using Student's t test.

**** $p < 0.00005$; *** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$. See also Figures S1 and S2 and Table S1.

Figure 2. Mannose Supplementation Changes Gut Microbial Composition

Schematic shows the experimental design. 16S rDNA sequencing was performed on 6–8 mice in each diet group: ND, HFD, or HFD+2% mannose where mannose was supplemented in water at 0 weeks (N0, H0, and HM0), 3 weeks (N3, H3, and HM3), or 8 weeks (N8, H8, and HM8) PW.

Changes in phyla with different diets. Significance was calculated for HFD+M versus HFD using Student's t test. ****p = 8.26E-10; ****p < 0.000005; ****p < 0.00005; ***p < 0.0005; **p < 0.005; *p < 0.05.

(C and D) The heatmaps generated using R Studio show the relative abundance of relevant genus (C) and species (D) in HFD and HFD+M groups. Percentage is the % identity to taxonomy (best hit) in NCBI 16S database. The accompanying box shows the color scale for relative abundance. Black color shows undetectable genera or species.

Also see Figure S3.

Figure 3. Mannose Modulates Transcriptome of Gut Microbiota

RNAs from cecal contents were pooled from 8 mice in each group weaned on HFD with mannose (HM) or without 2% mannose (H) in drinking water started at 3 and 8 weeks PW. Differential abundances of the contigs between HFD and HFD+M were calculated for both 3 weeks and 8 weeks PW. The comparisons were drawn between 3 weeks PW (mannose-responsive) mice and 8 weeks PW (non-responsive) mice. (A and B) Taxonomic analysis: comparison of the number of differentially abundant contigs mapped to various phyla (A) and genus (B).

(C–E) Functional analysis: heatmaps of relative transcript abundance based on RPKM values using R Studio. The accompanying box shows the color scale for relative abundance with red being the most abundant (set to a value of 1) and dark green being the least abundant. Black color in the heatmap depicts no differential abundance.

(C) Relative transcript distribution in various metabolic pathways.

(D and E) Relative transcript abundance of most relevant and abundant genes within the subsets of glycosyl hydrolases (D) and carbohydrate metabolism (E). Asterisk indicates the data for transcripts that did not meet the initial cutoff of 3 fold change; Kal 's $p < 10^{-5}$ for individual contigs, however, are shown for comparison. Also see associated Tables S2, S3, S4, S5, S6, S7, and S8.

(F) Summary of mannose effects and the potential mechanism.

Table 1.**Serum Analysis**

Serum Parameter	ND n = 8	HFD n = 8	HFD+M n = 8	t Test (HFD+M versus HFD)
Glucose (mg/dL)	245 ± 10	277 ± 9	238 ± 13	*
Triglycerides (mg/dL)	88 ± 4.4	71.6 ± 12.6	57.4 ± 2.8	ns
Free fatty acids (μmol/L)	827 ± 29	811 ± 27.1	771 ± 15.6	ns
Glycerol (mg/dL)	5.1 ± 0.25	5.2 ± 0.35	4.3 ± 0.22	*
Total cholesterol (mg/dL)	132 ± 7.1	155 ± 5.5	189 ± 2.6	***
VLDL/LDL (mg/dL)	23.3 ± 1.6	26.0 ± 2	26.6 ± 2.1	ns
HDL (mg/dL)	105 ± 6	116 ± 10.1	151.4 ± 6.9	*
Insulin (ng/mL)	1.3 ± 0.27	2.4 ± 0.56	1.26 ± 0.20	*
Adiponectin (μg/mL)	22.5 ± 1.4	31 ± 2	34.0 ± 2.5	ns
Leptin (ng/mL)	2.3 ± 0.4	6.54 ± 1.09	4.6 ± 1.2	ns

Mannose supplementation improves blood parameters of HFD mice. Data shown are mean ± SEM of 8 mice. Significance was calculated for HFD +M versus HFD using Student's t test.

p < 0.0005;

*
p < 0.05. See also Table S1.

Table 2.**Fecal Analysis**

Fecal Parameter	HFD	HFD+M	t Test
Energy content (KJ/g) n = 14	16.09 ± 0.18	17.94 ± 0.32	****
Mannose (μmol/g) n = 7–8	0.15 ± 0.06	6.0 ± 1.8	**
Total SCFA (μmol/g) n = 7–8	3.3 ± 0.4	7.6 ± 1.6	*
Acetate (μmol/g) n = 7–8	2.0 ± 0.4	5.9 ± 1.4	*
Propionate (μmol/g) n = 7–8	0.25 ± 0.04	0.5 ± 0.1	*
Butyrate (μmol/g) n = 7–8	0.18 ± 0.01	0.44 ± 0.07	*

Mannose supplementation increases fecal energy content, mannose, and SCFA. Data are mean ± SEM of indicated number of mice. Significance was calculated for HFD+M versus HFD using Student's t test.

p < 0.0001

**
p < 0.005

*
p < 0.05.