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Soy compared with milk protein in a Western diet changes fecal microbiota and decreases hepatic steatosis in obese OLETF rats

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Abstract

Soy protein is effective at preventing hepatic steatosis; however, the mechanisms are poorly understood. We tested the hypothesis that soy versus dairy protein-based diet would alter microbiota and attenuate hepatic steatosis in hyperphagic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Male OLETF rats were randomized to “Western” diets containing milk protein isolate (MPI), soy protein isolate (SPI), or 50:50 MPI/SPI (MS) (n=9–10/group; 21% kcal protein) for 16 weeks. SPI attenuated ($P<0.05$) fat mass and percent fat by ~10% compared with MS, but not compared with MPI. Serum TBAR and total and LDL-cholesterol concentrations were lower ($P<0.05$) with dietary SPI versus MPI and MS. Histological hepatic steatosis was lower ($P<0.05$) in SPI compared with MPI or MS. Lipidomic analyses revealed reductions ($P<0.05$) in hepatic diacylglycerols but not triacylglycerols in SPI compared with MPI, which was associated with lower hepatic de novo lipogenesis (ACC, FAS, and SCD-1 protein content, and hepatic 16:1 n-7

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Author contributions

Involved in the study concept and design (DNB, ESK, JPT, RSR); acquisition of data (MRP, CMS, KEP, GMM, SVC, UDW, KS, DNB, RSR); analysis and interpretation of data (MRP, KS, JPT, RSR); drafting of the manuscript (MRP and RSR); critical revision of the manuscript for important intellectual content (MRP, CMS, KEP, GMM, SVC, UDW, KS, DNB, ESK, JPT, RSR); statistical analysis (MRP, KS, DNB, ESK, RSR); obtained funding (KS, JPT, RSR). All authors have read and approved the final manuscript.

and 18:1 n-7 PUFA concentrations) ($P<0.05$) compared with MPI and MS; however, MPI displayed elevated hepatic mitochondrial function compared with SPI and MS. Fecal bacterial 16S rRNA analysis revealed SPI-intake elicited increases ($P<0.05$) in *Lactobacillus* and decreases ($P<0.05$) in *Blautia* and *Lachnospiraceae* suggesting decreases in fecal secondary bile acids in SPI rats. SPI and MS exhibited greater ($P<0.05$) hepatic *Fxr*, *Fgfr4*, *Hnf4a*, *HmgCoA* reductase and synthase mRNA expression compared with MPI. Overall, dietary SPI compared with MPI decreased hepatic steatosis and diacylglycerols, changed microbiota populations, and altered bile acid signaling and cholesterol homeostasis in a rodent model of obesity.

Keywords

soy protein; NAFLD; microbiota; bile acids; lipid metabolism; cholesterol

1. Introduction

Over 60% of the adult population in the United States is considered overweight or obese [1], which is accompanied by a concomitant increase in the incidence of nonalcoholic fatty liver disease (NAFLD). Approximately 30% of adults in the United States have NAFLD, with ~20% of obese individuals developing nonalcoholic steatohepatitis (NASH), an advanced liver phenotype of hepatic inflammation, and eventually fibrosis, and/or cirrhosis [2]. Clinical studies in adults and children have shown that alterations in the gut microbiome is involved with hepatic steatosis [2, 3]. To date, lifestyle modifications such as caloric restriction and exercise are most effective at preventing the onset of hepatic steatosis with very little evidence on the effects of diet composition.

Understanding the molecular mechanisms involved in development of hepatic steatosis and progression to a more severe liver phenotype is warranted. Recent findings suggest that dietary replacement of milk proteins with soy proteins and supplementation of soy bioactive components have beneficial effects on hepatic lipid metabolism and prevent hepatic steatosis [4–7]. Specifically, several mechanisms have been proposed linking soy protein-induced changes in hepatic steatosis through decreases in lipogenic gene expression [5], altered lipid trafficking to the liver by restoring Wnt/ β -catenin signaling [8] and decreased serum cholesterol through bile acid excretion and signaling [9]. Furthermore, dietary soy isoflavones, and in particular, genistein, is thought to be protective through improved mitochondrial function [10]. We previously reported in golden Syrian hamsters that dietary soy protein improves serum lipids compared with milk protein and these changes correlated with specific changes in the gut microbiota [11]; however, the molecular mechanisms by which soy improves metabolic outcomes remains largely unknown.

We have previously demonstrated that the hyperphagic Otsuka Long-Evans Tokushima Fatty (OLETF) rats (null expression of brain cholecystokinin A receptor), develop a NAFLD phenotype similar to that seen in humans [12]. Therefore, the objectives of this experiment were to (1) determine if a soy protein diet improves hepatic steatosis and lipid metabolism relative to a milk protein-containing diet and (2) evaluate whether dietary protein source-induced changes in the fecal microbiome are associated with improvements in hepatic

steatosis. We hypothesized that a soy protein diet would significantly attenuate hepatic steatosis development in hyperphagic OLETF rats in part through shifts in the fecal microbiome.

2. Methods

2.1 Animals and diets

Male OLETF rats (Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan); 4 wk of age) were individually housed in a 12h light, 12h dark cycle within temperature controlled animal quarters with *ad libitum* access to food and water throughout the duration of the experiment. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri and the Subcommittee for Animal Safety at the Harry S. Truman Memorial VA Hospital. Semi-purified “Western” diets were prepared by Research Diets, Inc., New Brunswick New Jersey, and are described in Table 1. All rats were fed a semi-purified diet (US17) containing 23g% protein, 49g% carbohydrate, and 17g% fat *ad libitum* for 1 week to acclimate. After this acclimation period rats were randomly assigned to 1 of 4 groups (n=9–10/group) and fed the same diet containing 23g% protein as milk protein isolate (MPI; Idaho Milk Products, Jerome, ID), soy protein isolate (SPI; DuPont Nutrition and Health, St. Louis, MO), or 50% MPI/50% SPI (MS) for an additional 16 wks. Each diet was formulated to be isonitrogenous and isocaloric on the basis of the guaranteed analysis provided by the manufacturer, thereby applying experimental focus strictly on the protein source. Daidzein, genistein and glycitein content of the SPI was 453, 731 and 62 µg/g protein (aglycone basis). The 50% MPI/50% SPI (MS) group was included to determine whether having a mixed protein source diet would be as effective as a purely plant-based protein source in improving physiologic outcomes.

Body mass, food intake, and body composition via EchoMRI (EchoMRI, Houston, Texas, USA) were assessed weekly. At completion of the intervention (20 weeks of age), animals were fasted for 5 hours and anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and exsanguinated by removal of the heart.

2.1.1 Serum and whole blood analyses—Fasting serum glucose (Thermo Scientific, Waltham, MA, USA), TG (Sigma, St. Louis, MO, USA), FFA (Wako Chemicals, Richmond, VA), insulin (EMD Millipore, Billerica, MA), total antioxidant (Cayman Chemical, Ann Arbor, MI), and TBAR (Cayman Chemical) were measured using commercially available kits. HDL cholesterol, LDL cholesterol, and total cholesterol were measured by Comparative Clinical Pathology Services (Columbia, MO, USA) using commercially available assays. Hemoglobin A1c (HbA1c) levels were measured in EDTA-treated whole blood with a DCA Vantage Analyzer (Seimens, Malvern, PA).

2.1.2 Tissue collection and mitochondria isolation—Livers were quickly excised from anaesthetized rats and either flash frozen in liquid nitrogen, placed in 10% formalin, or placed in ice-cold buffer (100 mM KCl, 40 mM Tris-HCl, 10 mM Tris-Base, 5 mM MgCl₂·6H₂O, 1 mM EDTA and 1 mM ATP; pH 7.4). Ileums were also excised (1 inch above ileal-cecal junction) and immediately flash frozen in liquid nitrogen. Hepatic

mitochondria were isolated using centrifugation procedures as previously described [13] and re-suspended in buffer MiPO3 (0.5 mM EGTA, 3 mM MgCl₂·6 H₂O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 g·L⁻¹ BSA, 20 mM histidine, 20 μM vitamin E succinate, 3 mM glutathione, 1 μM leupeptine, 2 mM glutamate, 2 mM malate, and 2 mM Mg-ATP).

2.1.3 Fatty acid oxidation—Fatty acid oxidation was performed in liver whole homogenates and isolated mitochondrial preparations using [1-¹⁴C] palmitate (American Radiolabeled Chemicals, St. Louis, MO) as previously described [14].

2.1.4 Hepatic mitochondrial respiration—Mitochondrial respiration was assessed using high resolution respirometry (Oroboros Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) as previously described [13]. Briefly isolated mitochondria were loaded into respiration chambers containing buffer MiR05 (100 mM sucrose, 60mM K-lactobionate, 0.5mM EGTA, 3mM MgCl₂, 20mM taurine, 10mM KH₂PO₄, 20mM HEPES, adjusted to pH 7.1 with KOH at 37°C; and 1 g·L⁻¹ fatty acid-free BSA) to assess basal respiration. Glutamate (5mM) and malate (2mM) were added to the chambers to assess State 2 respiration in the absence of ADP. State 3, complex I respiration was then assessed by titration of ADP (25–125mM). The addition of succinate (10mM) allowed for the measurement of State 3, complex I and complex II respiration. Finally, maximal uncoupled respiration was assessed by the addition of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, 0.25 μM.

2.1.5 Mitochondrial content—Citrate synthase and β- hydroxy-acyl-coenzyme A dehydrogenase (β -HAD) activities were determined using the methods of Srere [15] and Bass et al. [16], respectively, as previously described [14].

2.1.6 Intrahepatic lipid content and morphology—Formalin-fixed, paraffin-embedded livers and mixed gastrocnemius were sectioned and stained with hematoxylin and eosin to examine morphology. Triacylglycerols (TAGs) and diacylglycerols (DAGs) were extracted, separated by Thin-layer chromatography, scraped, methylated, and quantified by gas chromatography as previously described by our group [14].

2.1.7 Quantitative Lipidomics—Lipidomic analyses were performed liver samples as described by Cao et al. [17] at Metabolon, Durham, NC. Data were analyzed using the Metabolync™ software (Metabolon, Durham, NC).

2.1.8 Western Blots—Protein content for Apo B100 (Abcam, Cambridge, MA), cluster of differentiation 36 (CD36) (Santa Cruz Biotechnology, Inc., Dallas, TX), fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), phospho-ACC ser79 (Cell Signaling Technology, Danvers, MA), and stearoyl CoA desaturase 1 (SCD-1) (Alpha Diagnostic International, San Antonio, TX) were determined by western blot as previously described [14].

2.1.9 Liver and ileal gene expression—Frozen livers and ileums were first homogenized using a TissueLyzer II (Qiagen; Valencia, CA) and RNA was isolated from rats via a commercially available kit (RNeasy Mini Kit, no. 74104, Qiagen). RNA purity was

determined using a Nanodrop spectrophotometer (Nanodrop 2000c, Thermo Scientific, Waltham, MA), and cDNA was synthesized via reverse transcriptase (ProMega, Madison, WI). Real-time quantitative PCR was performed with the ABI 7500 Fast Sequence Detection System (Applied Biosystems, Carlsbad, CA) using Fast SYBR Green Master Mix (Applied Biosystems). Supplemental table 1 displays the primer pairs and forward and reverse sequences for all genes measured. Gene specific values were normalized to peptidylprolyl isomerase B for liver and ribosomal protein S18 for ileum. Dissociation melt curves were analyzed to verify primer specificity. Liver and ileal mRNA expression of peptidylprolyl isomerase B and ribosomal protein S18 was used to calculate the expression levels of genes of interest using the 2^{-CT} method, respectively. All data are normalized to expression levels in MPI-fed group.

2.2 Hepatic glycogen and glutathione content

Hepatic glycogen was assessed as previously described by our group [14]. Reduced (GSH) and oxidized (GSSG) glutathione concentrations were determined by a fluorometric method as previously described by our group [12].

2.2.1 Fecal DNA extraction and MiSeq Illumina sequencing—Microbial DNA was isolated from fecal contents using QIAamp Fast DNA stool mini kit (Qiagen) including a bead beating step. Genomic DNA was utilized for amplification of the V4 variable region of the 16S ribosomal RNA gene using 515F/806R primers. Primers were dual-indexed as described by Kozich et al [18] to accommodate multiplexing 384 samples per run. Paired-end sequencing (2×250 bp) was carried out using Illumina Miseq platform. Processing and quality filtering of reads was performed by using scripts in Quantitative Insights into Microbial Ecology (QIIME) (v1.9.1) [19, 20] and other scripts. Paired reads were stitched with paired-end read merger [21] and further filtered based on Phred quality scores ($Q > 19$) and for chimeric reads using USEARCH61 [20, 22]. Filtered reads were demultiplexed within QIIME and samples with less than 5000 reads were excluded from further analysis. UCLUST was used to cluster sequences into operational taxonomical units (based on 97% identity) [22]. Operational taxonomic unit picking was performed using open-reference method, which encompasses clustering of reads against a reference sequence collection and also performs *de novo* operational taxonomic unit picking on the reads which fail to align to any known reference sequence in the database [23]. To eliminate erroneous mislabeling, the resulting operational taxonomic unit tables were checked for mislabeling sequences [24]. Representative sequences were further aligned using Python Nearest Alignment Space Termination with the Greengenes core-set alignment template [25]. Construction of the phylogenetic tree was performed using the FastTree software method in QIIME [26]. Alpha rarefaction was performed using the phylogenetic diversity, Chao1 and observed species metrics. Beta diversity estimation was carried out by computing weighted and un-weighted UniFrac distances between samples using QIIME [27]. All samples were clustered based on their between-sample distances using Unweighted Pair Group Method with Arithmetic Mean, and subsequent jackknifing was performed by resampling methods. Comparisons of intergroup and intragroup diversity were performed using ANOVA including correction for multiple comparisons using Statistical Analysis of Metagenomic Profiles.

2.2.2 Statistical analysis—All data were analyzed as ANOVA using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Means were separated for diets using a Fisher-protected least significant difference. Post-hoc Pearson's correlations were analyzed using GraphPad Prism 6 (La Jolla, CA). Significant differences between dietary groups were set at P 0.05.

3. Results

3.1 Animal and serum characteristics

Animal characteristics and serum outcomes are presented in Table 2. Final body weight and lean body mass were not different among dietary treatments. Total fat mass and percent body fat were lower (P 0.05) in SPI fed rats compared with MS fed rats, but not MPI fed rats. In addition, no differences were observed in fat pad depots examined (omental, epididymal, and retroperitoneal; data not shown), suggesting the lower percent body fat detected by EchoMRI between SPI and MS rats was either due to other visceral fat depots or differences in subcutaneous fat. Both MS and SPI rats had greater (P 0.05) liver weight compared with MPI fed rats, while SPI-fed rats had greater (P 0.05) liver glycogen concentrations compared with MPI-fed rats. Weekly food intake was greater (P 0.05) in MS-fed rats compared with MPI-fed rats.

Serum triglycerides, FFA, and glucose did not differ among groups; however, serum insulin and HbA1c were significantly elevated in SPI compared with MPI (P <0.01). Soy protein isolate feeding resulted in significant reductions in total cholesterol, LDL-C, and serum TBAR (SPI significantly lower than MS and MPI groups, P <0.05).

3.1.1 Hepatic steatosis and intrahepatic TAG and DAG content—Representative hematoxylin and eosin stained liver sections, and liver TAG and DAG concentrations for MPI, MS, and SPI fed rats are shown in Figure 1. Interestingly, total hepatic TAG content did not differ among groups (Figure 1A); however, total hepatic DAG content was significantly reduced in SPI compared with MPI rats (P <0.01, Figure 1B). The relative proportion of PUFA, n-3, and n-6 fatty acids was significantly increased (P <0.05), while reductions (P <0.05) in the proportion of n-7 (i.e. 16:1 n-7 and 18:1 n-7) hepatic TAGs were observed in the SPI-fed group (P <0.05) compared with the MS and MPI-fed groups. The relative proportions of saturated, MUFAs and n-7 fatty acids in the hepatic DAGs were significantly reduced (P <0.05) in SPI compared with MS and MPI rats. In addition, the proportion of n-9 fatty acids was significantly reduced in the hepatic DAGs in the SPI compared with the MS and MPI fed groups.

3.1.2 Measure of hepatic mitochondrial function and content—In order to examine the potential mechanisms by which soy protein isolate reduced hepatic steatosis and hepatic DAG content, we assessed a number of measures of hepatic mitochondrial content and function. Surprisingly, animals fed MPI had higher hepatic mitochondrial respiration compared with MS and SPI groups (P <0.05, Figure 2A). In addition, MPI fed OLETF rats also had elevated hepatic β -HAD activity in isolated mitochondria and whole liver lysate (Figure 2B and C) and hepatic citrate synthase activity in isolated mitochondria compared with MS and SPI groups (P <0.05, Figure 2D). Citrate synthase activity in whole liver lysate

exhibited a trend to be greater in MPI fed OLETF rats compared with MS and SPI-fed rats ($P=0.10$, Figure 2E). Complete (to CO_2), incomplete (ASM), and total (acid soluble metabolites + CO_2) palmitate oxidation in isolated mitochondria and whole liver lysate (Complete oxidation shown Figure 2F & G; incomplete and total data not shown) was not different among groups. Furthermore, we found no differences in hepatic Peroxisome proliferator-activated receptor gamma coactivator 1-alpha or Sirtuin 1 mRNA expression or protein content among groups (data not shown).

3.1.3 Markers of hepatic de novo lipogenesis, fatty acid uptake and fatty acid export—Soy protein isolate feeding dramatically reduced ($P<0.01$) hepatic ACC, phospho ACC, FAS, and SCD-1 protein content compared with MS and MPI rats (Figure 3A–D). In addition, the marker of fatty acid transport, CD36, did not differ among feeding groups (Figure 3E), but soy protein isolate increased hepatic Apo B100 protein content, a marker of hepatic triglyceride export, by ~40% compared with MPI and MS rats ($P<0.05$, Figure 3F).

3.1.4 Hepatic markers of inflammation and oxidative stress—There was no robust hepatic inflammatory cell infiltration as revealed by hematoxylin and eosin staining, hepatic fibrosis by trichrome staining, or hepatic mRNA expression of inflammatory markers (Toll-like receptor (*Tlr4*), *Tnfa*, *Il1beta*, or *Cd11c*) in the 3 feeding groups (data not shown). Hepatic glutathione peroxidase 1 mRNA was significantly elevated in the MS group compared with MPI and SPI ($P<0.05$, Figure 4A). In addition, hepatic monocyte chemoattractant protein-1 mRNA expression was significantly attenuated in SPI compared with MPI rats ($P<0.05$, Figure 4B). Liver GSH exhibited a trend ($P=0.07$) with SPI-fed rats having elevated GSH compared with MPI (Figure 4C). Furthermore, both MS and SPI had lower GSSG and a GSH/GSSG ratio compared with MPI rats ($P<0.05$, Figure 4D and E).

3.1.5 Microbiota analysis and Pearson's correlations—Taxonomic classification of raw sequences by Ribosomal Database Project classifier were assigned to 7 phyla, 28 families, and 38 genera. Approximately 99% of all sequences were assigned to the phyla of Bacteroidetes, Firmicutes, and Proteobacteria, while the remaining sequences were assigned to Deferribacteres, Verrucomicrobia, and Tenericutes. The most abundant phyla were Bacteroidetes (52.1% of total sequences), Firmicutes (23.0% of total sequences), and Proteobacteria (17.8% of total sequences) in rats provided MPI, MS, and SPI. Mean relative operational taxonomic unit abundances were not affected by diet at the phylum level (Supplemental Table 2).

Several changes at the family and genus taxonomic levels were noticed (Supplemental Table 2). Fecal relative abundances of unassigned families within the Bacteroidetes phylum were greater ($P<0.05$) in rats fed MPI and MS compared with SPI fed rats. *Prevotellaceae* were lower ($P<0.05$) in feces of MPI and MS fed rats compared with SPI fed rats, while fecal *S24-7* relative abundance was lower ($P<0.05$) in MPI fed rats compared with both MS and SPI rats. Within Firmicutes, fecal relative abundances of unassigned Clostridia were decreased ($P<0.05$) in rats fed milk protein compared with SPI fed rats, while the relative abundance of *Blautia* was increased ($P<0.05$) in MPI fed rats compared with MS and SPI diets. Members of the Proteobacteria phylum, including *Alicalignaceae*, and specifically

Sutterella were lower ($P<0.05$) in relative abundance in rats fed milk protein-containing diets compared with the SPI diet.

Indices of alpha diversity were measured using Chao 1 index, observed operational taxonomic units, and phylogenetic diversity Whole Tree (Supplementary figure 1A–C). All indices of alpha diversity were greater ($P<0.05$) in rats fed SPI vs. MPI. No significant differences were observed in species richness or microbial diversity between MS- and SPI-fed rats. Rats fed MS had greater ($P<0.05$) species richness and observed operational taxonomic units compared with MPI-fed rats. Figure 5 displays beta diversity as plotted by unweighted Unifrac distances on a principal coordinates analysis plot. Rats provided MPI were more similar to each other than to both MS and SPI-fed rats ($P<0.05$ as measured by two-sample Monte Carlo t-test). MS and SPI-fed rats showed no significant differences in beta diversity. Weighted Unifrac distances revealed no significant clustering between any of the diets (data not shown).

Figure 6 displays fecal microbiota impacted by the experimental diets and correlations with serum cholesterol concentrations. Specifically, fecal *Lachnospiraceae* (Figure 6A) exhibited a tendency ($P=0.095$) to be greater in MPI-fed rats compared with soy-containing diets. Fecal relative abundance of *Blautia* (Figure 6B) was greater ($P<0.05$) in MPI-fed rats compared with MS and SPI diets. MPI-fed rats had lower ($P<0.05$) relative abundance of fecal *Oscillospira* (Figure 6C) compared with MS- and SPI-fed rats, while MPI- and MS-fed rats had lower ($P<0.05$) fecal relative abundance of *Lactobacillus* compared with SPI-fed rats (Figure 6D). Pearson's correlations revealed a significant negative correlation between serum total cholesterol concentrations and *Lactobacillus* (Figure 6E; $r=-0.56$; $P<0.01$). Specifically, rats fed soy-containing diets (i.e. MS and SPI diets) displayed a tendency for a negative correlation between serum cholesterol concentrations and *Lactobacillus* (Figure 6F; $r=-0.46$; $P=0.07$).

3.1.6 Ileal gene expression and hepatic markers of cholesterol/bile acid synthesis and signaling

Ileal gene expression of zona occludin-1 (*Zo1*) was greater ($P<0.05$) in SPI- and MS-fed rats compared with MPI-fed rats (Figure 7A). However, no significant differences were noticed in ileal gene expression of zona occludin-2 (*Zo2*) or for the SCFA receptor free fatty acid receptor 2 (*Ffar2*) or the bile acid receptor farnesoid X receptor (*Fxr*) (Figure 7B–D). We assessed hepatic gene markers of enzymes involved in bile acid and cholesterol synthesis (Figure 8A–F), bile acid receptors (Figure 8G and H), and downstream targets of *Fxr* and fibroblast growth factor receptor 4 (*Fgfr4*) (Figure 8I–K). Bile acid synthesis enzyme cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*) (Figure 8A) hepatic mRNA expression was not different among dietary treatments; however, hepatic cytochrome P450 family 7 subfamily B member 1 (*Cyp7b1*) mRNA expression was greater ($P<0.05$) in both soy protein-containing diets compared with MPI (Figure 8B). No differences were observed in hepatic hydroxysteroid (17-beta) dehydrogenase 2 (Figure 8C) gene expression among dietary treatments. Hepatic 3-Hydroxy-3-Methylglutaryl CoA (*HmgCoA*) synthase (Figure 8D, $P<0.05$) mRNA expression was increased in rats fed the SPI diets compared with MPI-fed rats, and *HmgCoA* reductase (Figure 8E) tended ($P=0.06$) to be increased in SPI-fed rats compared with MS and MPI-fed rats. In addition, hepatic squalene epoxidase (Figure 8F) mRNA expression was greater ($P<0.05$) in SPI rats

compared with MPI rats. Rats fed soy-containing diets had greater hepatic mRNA expression ($P<0.05$) of bile acid receptors *Fxr* and *Fgfr4* (Figure 8G and H) compared with MPI-fed rats. Hepatic gene expression of hepatic nuclear factor alpha (*Hnf4a*) (Figure 8I) tended ($P=0.07$) to be elevated with SPI and MS feeding; however, no significant changes were observed in small heterodimer partner or proprotein convertase subtilisin/kexin type 9 (Figure 8J and K) mRNA expression.

4. Discussion

NAFLD is linked to poor dietary choices [28]; however, the mechanisms and clinical implications underlying this relationship are not fully understood. On the other hand, increased consumption of dietary soy protein is associated with decreased cardiovascular disease, type 2 diabetes, and NAFLD [29–31]. Here, we demonstrate that dietary soy protein in the obese, hyperphagic OLETF rat decreased hepatic steatosis, lowered lipid species known to be bioactive (DAGs), changed microbiota populations, induced evidence of alterations in bile acid signaling, and improved cholesterol homeostasis compared with a milk protein source diet.

Ecological measures of both alpha and beta diversity were significantly different among diets, particularly alpha diversity/species richness was greater in SPI-fed rats compared with MPI-fed rats. Our data also support a dose response with dietary SPI on microbiota species richness and show a distinct microbiome from MPI rats, which is consistent with previous work in hamsters [11]. This is important and in line with previous observations in children with obesity and NASH that exhibit microbial dysbiosis and lower species richness compared with healthy controls [2]. The differences in the microbial profiles of the soy fed groups may be due to differences in how the protein components affect bile acid metabolism [32–34] or antimicrobial activities of dietary protein–derived peptides [35–38], or perhaps the differences may be due to the influence of the higher isoflavones in the soy diet [39]. Determining whether isoflavones or differences in amino acid composition are the mechanism responsible for the changes in microbial composition with soy protein isolated feeding warrants future investigation.

Here we found that soy protein diets elicited increases in fecal *Oscillospira*, a known fiber degrader and short-chain fatty acid (SCFA) producer [40], compared with MPI-fed rats. However, ileal *Ffar2* gene expression, a receptor for SCFA, showed no change. Although the physiological relevance has not been determined, fecal *Oscillospira* was reported to be lower in children and obese adults with NASH and NAFLD compared with healthy controls [2]. Soy protein diets also increased the fecal relative abundance of *Lactobacillus* and increased ileal *Zo1* expression, as well as lowered hepatic *de novo* lipogenic markers compared with MPI. Enzymatic activity of *Lactobacillus* through beta-galactosidase and glucosidase can increase the absorption of isoflavones [41], which deconjugates isoflavone-glycosides to their more bioavailable aglycone forms. Genistein also may act as an antioxidant source to tight junction proteins by blocking tyrosine phosphorylation induced by cytokines, enteric bacteria, and acetaldehyde [42]. In addition, daidzein supplementation has been shown to lower hepatic steatosis and markers of *de novo* lipogenesis [43]. Overall, SPI intake increased fecal *Lactobacillus*, which likely promotes increased isoflavone absorption and

resulted in the increase in Zo1 expression and potentially improved intestinal integrity. These possibilities warrant future, more mechanistic examination.

Soy protein-containing diets also lowered serum total and LDL cholesterol, which were negatively correlated with fecal *Lactobacillus*. Interestingly, SPI feeding increased cholesterol synthesis genes (*HmgCoA* synthase, *HmgCoA* reductase, and squalene epoxidase mRNA expression), suggesting a feedback regulation of cholesterol synthesis. Dietary pre and/or probiotic supplements, as well as plant sterols and grain sorghum have been shown to lower cholesterol concentrations in connection with the microbiota [44–46]. In addition, we have reported similar findings with soy protein feeding in Syrian hamsters [11]. Dietary soy protein also resulted in lower circulating byproduct of lipid peroxidation (TBAR), hepatic oxidized glutathione, and increased hepatic reduced:oxidized glutathione ratio. This is consistent with previous studies demonstrating antioxidative effects of dietary soy versus milk protein [47, 48]. Despite improvements in cholesterol homeostasis and reduced indices of oxidative stress with soy protein feeding, serum insulin (5 hr fasted) and HbA1c levels were elevated with SPI compared with MPI feeding. While no dynamic glycemic challenge measures were performed, such as GTTs, ITTs or hyperinsulinemic-euglycemic clamps, these findings may indicate early signs of insulin resistance with soy protein feeding. This is in contrast to previous studies in other rat models that have shown improvements in glycemic control in response to soy protein feeding [49, 50]. However, these previous investigations were not on a western diet background and utilized different animal models. More thorough examination in this area is warranted to determine the cause and the potential consequences of this outcome. Collectively, dietary soy protein lowered serum cholesterol and markers of oxidative stress without improving markers of glycemic control compared with milk protein, suggesting an overall healthier cardiometabolic profile.

Limited evidence suggests that soy protein reduces hepatic TAG concentrations through increasing hepatic mitochondrial function [51–53]. Whereas, non-fat dry milk protein with calcium supplementation vs soy protein based diets also elicited decreases in liver TAGs [54, 55]. Here we found that SPI-fed rats exhibited reduced markers of hepatic mitochondrial content and function compared with MPI-fed rats. However, histological analysis revealed lower hepatic steatosis and lipid vacuolization and reduced total liver biochemical DAGs with dietary SPI feeding. SPI feeding also increased total, n3, and n6 PUFAs in hepatic TAGs and decreased n7 PUFAs. Both n3 and n6 PUFAs activate peroxisome proliferator activated receptors and inhibit *de novo* lipogenic activators liver X receptor and sterol regulatory element-binding protein [56]. SPI feeding also lowered delta-9 desaturase, which mediates 18:1 n7 production [57], consistent with our observed reduction in n7 PUFA species. Interestingly, *Cd36* mRNA expression (a marker of fatty acid uptake) did not differ among groups; however, SPI intake increased hepatic Apo B100 content, suggesting increased capacity for hepatic TAG export and possibly TAG turnover in the liver. While no differences were observed in total hepatic TAG content, these data suggest that dietary soy protein curbed hepatic *de novo* lipogenesis, increased machinery for VLDL-TAG export, and improved lipid remodeling in the liver by doubling PUFA content in TAGs and lowering total and saturated fatty acids in DAGs.

Microbial metabolism of bile acids has been linked to hepatic lipid and cholesterol metabolism [58–60]. The observed changes in the fecal microbiota in the current report suggest an impact on enterohepatic circulation and bile acid metabolism. Islam et al. [61] fed bile acids to rats and found a higher prevalence of Firmicutes with 7 α -dehydroxylating activity. Here, rats fed MPI exhibited increases in the family *Lachnospiraceae* and genus *Blautia*, which have *Clostridium* cluster XIVa species (i.e. *Clostridium* and *Ruminococcus* spp.) that dehydroxylate bile acids at the 7 α carbon to the relatively more bacteriocidal secondary bile acid, deoxycholic acid [60–62]. In contrast, dietary SPI elicited increases in fecal *Lactobacillus*, which contains species with bile salt hydrolase activity [63]. Although the health implications are not fully understood, it is suggested that a bile salt hydrolase may have cholesterol-lowering effects through decreasing cholesterol absorption in the gut [63, 64]. These outcomes are consistent with our observation of lower serum cholesterol in SPI fed OLETF rats.

We investigated whether changes in the fecal microbiome corresponded with ileal and hepatic bile acid signaling and found that SPI feeding increased hepatic expression of the bile acid-activated gene *Fxr* and *Fgfr4* (receptor for intestinally derived fibroblast growth factor 15/19). Interestingly, hepatic *Cyp7b1* gene expression increased in rats fed soy diets. This may be due to increased *Hnf4a* expression, which upregulates bile acid synthetic gene expression parallel to *Fxr* activation [65]. Both *Fxr* and *Fgfr4* activation are known to reduce hepatic lipid and cholesterol synthesis, gluconeogenesis, hepatic fibrosis and increase glycogen storage [66–68]. In this study, the SPI diet lowered total and LDL-cholesterol, lipogenic gene expression, hepatic DAGs and liver glycogen, which are consistent with *Fxr* and *Fgfr4* activation. These data collectively suggest that SPI lowered hepatic *de novo* lipogenesis through modulation of hepatic FFA species and also through altered bile acid signaling.

In conclusion, this study provides evidence that soy protein isolate feeding lowers hepatic lipid accumulation and DAG content compared with milk protein containing diets in obese OLETF rats fed a western-style diet. Soy protein-containing diets increased gut microbial diversity and altered microbial composition consistent with upregulation in bile acid signaling pathways. In addition, soy protein consumption elicited increases in the relative proportion of polyunsaturated hepatic fatty acids compared to the milk protein fed group. These changes support our observed decreases in hepatic *de novo* lipogenesis and circulating cholesterol concentrations. Overall, the link between soy protein isolate intake and improved liver lipid metabolism may be due to differences in fatty acid and bile acid metabolism and signaling linked to the gut microbiota.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Soy protein decreased hepatic steatosis and hepatic DAG content.
- Soy protein suppressed hepatic de novo lipogenesis.
- Soy protein increased fecal *Lactobacillus* that was linked to lower serum cholesterol.
- Soy protein elicited beneficial changes in the fecal microbiota.
- Soy protein feeding altered markers of bile acid signaling.

Figure 1.

Liver hematoxylin and eosin stains of rats fed diets containing milk protein isolate (MPI), 50/50 milk and soy protein isolates (MS), and soy protein isolate (SPI) (A). Biochemical triacylglycerol (TAG) (B) and diacylglycerol (C) concentrations were measured in rats fed MPI, MS, and SPI. All values are means $\pm$ SE. The n7 fraction consists of the species 16:1 and 18:1 n7. Means with different letters denote significant differences ($P < 0.05$).

Figure 2.

Hepatic mitochondrial respiration (A), β -HAD and citrate synthase activity in isolated mitochondria (B and C) and whole liver lysate (D and E), complete palmitate oxidation of isolated mitochondria (F) and whole liver lysate (G) in rats fed diets containing milk protein isolate (MPI), 50/50 milk and soy protein isolates (MS), and soy protein isolate (SPI). All values are means $\pm$ SE. Means with different letters denote significant differences ($P < 0.05$).

Figure 3.

Liver protein content of enzymes involved in hepatic de novo lipogenesis (acetyl CoA carboxylase (ACC) (A), phosphorylated ACC (B), fatty acid synthase (FAS) (C), stearoyl-CoA desaturase 1 (SCD1) (D) and fatty acid transport (CD36 (E) and Apolipoprotein (Apo) B100 (F) in rats fed diets containing milk protein isolate (MPI), 50/50 milk and soy protein isolates (MS), and soy protein isolate (SPI). All values are means $\pm$ SE. Means with different letters denote significant differences ($P < 0.05$). Representative western blot images are shown in panel G.

Figure 4.

Hepatic gene expression of monocyte chemoattractant 1 (*Mcp1*) (A) and *Gpx1* (B), and hepatic oxidized glutathione (GSH) (C), reduced glutathione (GSSG) (D), and the ratio of GSH/GSSG (E) in rats fed diets containing milk protein isolate (MPI), 50/50 milk and soy protein isolates (MS), and soy protein isolate (SPI). All values are means $\pm$ SE. Means with different letters denote significant differences ($P < 0.05$).

Figure 5. Unweighted UniFrac distances of fecal microbiota in rats fed milk protein isolate (MPI; red circles), 50/50 milk and soy protein isolate (MS; blue circles), and soy protein isolate (SPI; orange circles). Significant ($P < 0.05$) differences were observed between MPI versus MS and SPI groups using a two-sample Monte Carlo t-test.

Figure 6. Fecal relative abundances of *Lachnospiraceae* (A), *Blautia* (B), *Oscillospira* (C), *Lactobacillus* (D) in rats fed milk protein isolate (MPI), 50/50 milk and soy protein isolate (MS), and soy protein isolate (SPI). Values are means $\pm$ SE. Means with different letters denote significant differences ($P<0.05$). Pearson's correlation with all dietary treatments (E) and soy protein-containing dietary treatments (i.e. only MS and SPI groups) (F) between *Lactobacillus* and serum cholesterol concentrations. Circles; MPI, triangles; MS, and squares; SPI.

Figure 7.

Ileal gene expression of zona occludin (*Zo*) 1 (A), *Zo2* (B), free fatty acid receptor 2 (*Ffar2*) (C), and farnesoid X receptor (*Fxr*) (D) in rats fed milk protein isolate (MPI), 50/50 milk and soy protein isolate (MS), and soy protein isolate (SPI) diets. Values are means $\pm$ SE. Means with different letters denote significant differences (P<0.05)

Figure 8.

Liver gene expression of enzymes involved in bile acid and cholesterol synthesis, bile acid receptors, and downstream targets of bile acid receptors. Hepatic mRNA expression of cholesterol 7 alpha-hydroxylase (*Cyp7a1*) (A), oxysterol 7 alpha-hydroxylase (*Cyp7b1*) (B), Hydroxysteroid (17-Beta) Dehydrogenase 2 (*Hsd17b2*) (C), 3-hydroxy-3-methylglutaryl-CoA (*HmgCoA*) synthase (D) and reductase (E), Squalene epoxidase (F), *farnesoid X receptor* (*Fxr*) (G), fibroblast growth factor 4 receptor (*Fgfr4*) (H), hepatic nuclear factor-4alpha (*Hnf4a*) (I), proprotein convertase subtilisin/kexin type 9 (*Pcsk9*), and small heterodimer partner (*Shp*) (D) in rats fed milk protein isolate (MPI), 50/50 milk and soy

protein isolate (MS), and soy protein isolate (SPI) diets. Values are means $\pm$ SE. Means with different letters denote significant differences ($P < 0.05$).

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TABLE 1Ingredient and nutrient composition of experimental diets¹

Ingredient	Diet, g/kg		
	MPI	MS	SPI
Cornstarch	240	240	240
Total Milk Protein Isolate-MPI ²	217.5	108.8	0
Soy protein isolate ³	0	100	200
Sucrose	100	100	100
Maltodextrin	75	75	75
Palm oil, bleached, deodorized	52.5	52.5	52.5
Cellulose	50	50	50
Cocoa butter, deodorized	37.5	37.5	37.5
Safflower oil, USP	28.5	28.5	28.5
Sunflower oil	27	27	27
Potassium citrate	16.5	16.5	16.5
Dicalcium phosphate	13	13	13
Mineral mix	10	10	10
Vitamin mix	10	10	10
Calcium carbonate	5.5	5.5	5.5
Linseed oil	4.5	4.5	4.5
DL-methionine	3	3	3
Choline bitartrate	2	2	2
t-Butylhydroquinone	0.03	0.03	0.03
Protein, % energy	21	21	21
Carbohydrate, % energy	44	44	44
Fat, % energy	35	35	35

¹ Milk protein isolate and soy protein isolate were added to achieve equal macronutrient concentrations between experimental diets. The nutrient composition of all experimental diets was as follows: protein, 23.2g%; carbohydrate, 48.6g%; fat, 17.1g%; energy, 4.41 kcal/g.

² MPI, Milk Protein Isolate; MPI-85, Idaho Milk Products

³ Soy Protein Isolate; SUPRO@670, DuPont Nutrition & Health

TABLE 2

Animal characteristics and serum outcomes in OLETF rats fed diets containing milk protein (MPI), 50/50 milk and soy protein (MS), and soy protein isolate (SPI) for 16 wk.

Animal characteristics	Diet		
	MPI	MS	SPI
Body weight, g	651.2 ± 15.1	686.7 ± 8.10	652.0 ± 14.1
Food intake, g/wk	170.4 ± 2.90 ^a	181.0 ± 2.30 ^b	174.0 ± 2.70 ^{a,b}
Fat mass, g	230.7 ± 11.6 ^{a,b}	253.8 ± 7.70 ^b	219.9 ± 7.70 ^a
Lean mass, g	385.1 ± 4.90	393.3 ± 4.80	394.0 ± 5.40
Body fat, %	35.3 ± 2.70 ^{a,b}	36.9 ± 2.60 ^b	33.6 ± 2.70 ^a
Liver mass, g	20.9 ± 0.70 ^a	23.4 ± 0.60 ^b	23.1 ± 0.40 ^b
Liver mass, mg/g BW	32.1 ± 0.68 ^a	34.1 ± 0.76 ^b	35.4 ± 0.43 ^b
Liver glycogen, mg/g	23.3 ± 2.20 ^a	29.1 ± 2.20 ^{a,b}	33.2 ± 2.30 ^b
Serum characteristics			
Triglyceride, mg/dL	150.6 ± 12.4	197.4 ± 23.8	161.7 ± 11.7
Free fatty acid, uM/L	263.0 ± 21.2	276.2 ± 31.0	218.0 ± 15.4
Insulin, ng/mL	12.1 ± 0.80 ^a	15.6 ± 0.70 ^b	16.6 ± 1.00 ^b
Glucose, mg/dL	265.9 ± 26.4	280.0 ± 24.6	299.6 ± 16.5
Hemoglobin A1C, %	4.38 ± 0.08 ^a	5.24 ± 0.36 ^{ab}	5.94 ± 0.29 ^b
Total Cholesterol, mg/dL	182.2 ± 7.50 ^a	170.8 ± 4.90 ^a	142.0 ± 5.30 ^b
LDL-C, mg/dL	47.4 ± 1.00 ^a	48.8 ± 1.10 ^a	43.0 ± 1.50 ^b
HDL-C, mg/dL	19.6 ± 1.10	19.8 ± 0.90	18.0 ± 0.90
Antioxidant capacity, mM	0.47 ± 0.03	0.52 ± 0.03	0.53 ± 0.01
TBARS, μM	11.8 ± 0.70 ^a	11.3 ± 0.4 ^a	9.70 ± 0.50 ^b

Values are presented as means ± SEM (n= 6–10/ group), MPI, milk protein isolate; MS, 50% milk protein/50% soy protein; SPI, soy protein isolate. Superscripts with different letters indicate significant differences (P < 0.05). Low density lipoprotein-cholesterol, LDL-C; High density lipoprotein-cholesterol, HDL-C; thiobarbituric acid reactive substances; TBARS