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Soy Protein Compared with Milk Protein in a Western Diet Increases Gut Microbial Diversity and Reduces Serum Lipids in Golden Syrian Hamsters[1–3]

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Abstract

Background: Diet is a major factor influencing the composition and metabolic activity of the gut microbiota.

Objective: This study investigated the effect of soy compared with dairy protein on the gut microbiota of hamsters to determine whether changes in microbiota could account for soy protein's lipid lowering properties.

Methods: Thirty-two 6- to 8-wk-old, male Golden Syrian hamsters were fed a Western diet containing 22% (%wt) milk protein isolate (MPI) as the single protein source for 3 wk followed by 6 wk of one of 4 diets containing either [22% protein (%wt)]: MPI, soy protein concentrate (SPC), partially hydrolyzed soy protein isolate (SPI1), or intact soy protein isolate. Serum lipids, hepatic gene expression, and gut microbial populations were evaluated.

Results: Serum total and LDL-cholesterol concentrations were lower in the SPC-fed group (183 ± 9.0 and 50 ± 4.2 mg/dL, respectively) than in the MPI group (238 ± 8.7 and 72 ± 3.9 mg/dL, respectively) ($P < 0.05$). Triglyceride (TG) concentrations were lower ($P < 0.05$) in the SPI1-fed group (140 ± 20.8 mg/dL) than in the MPI-fed group (223 ± 14.2 mg/dL). VLDL and non-HDL-cholesterol concentrations were lower (by 40–49% and 17–33%, respectively) in all soy-fed groups than in the MPI-fed group ($P < 0.05$). Sequencing of the 16S ribosomal RNA gene revealed greater microbial diversity in each soy-fed group than in the MPI-fed group ($P < 0.05$). The cholesterol- and TG-lowering effect of soy protein was associated with higher expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), lanosterol synthase (*Lss*), and farnesyl-diphosphophosphate farnesyl-transferase 1 (*Fdft1*) (1.6–2.5-fold higher), and lower steroyl-CoA desaturase-1 (*Scd1*) expression (37–46% lower) in all soy-fed groups ($P < 0.05$) compared with the MPI-fed group. Gut microbes that showed significant diet differences were significantly correlated ($\rho = -0.68$ to 0.65, $P < 0.05$) with plasma lipids and hepatic gene expression.

Conclusion: Dietary protein sources in male Golden Syrian hamsters fed a Western diet affect the gut microbiota, and soy protein may reduce lipogenesis through alterations of the gut microbial community. *J Nutr* 2016;146:697–705.

Keywords: hamster, soy, protein, gut microbiota, lipid metabolism, cholesterol, gene expression

Introduction

Human health is largely dependent upon diet, a substantial effect of which is mediated through changes in the composition of the gut microbiota (1–4). Studies show that the gut microbiome can rapidly respond to dietary changes, particularly when transitioning between plant- and animal-based diets (5). Interestingly, functional profiles of the altered microbial populations correlate significantly with metabolic activities that leverage the altered luminal environment (5).

Few studies have specifically evaluated the impact of a protein source on the composition of the gut microbiota. Faith et al. (6) showed that feeding mice increasing proportions of casein protein altered microbial profiles. In rats, substituting ~20% of the casein protein in a cholesterol-enriched AIN76 diet with soy protein from freeze-dried soymilk for 6 wk resulted in an increased fecal *Firmicutes*-to-*Bacteroidetes* ratio due to increased *Lactobacillus* spp; soy protein also reduced both fecal *Allobaculum* spp. and *Parabacteroides*, which were elevated in rats fed the cholesterol-enriched AIN76 diet alone (7). An et al. (8) evaluated the effects of casein, soy protein, or fish meal on the gut microbiota in rats after 16 d of feeding. In that study, microbial diversity in cecal samples was significantly higher for rats fed soy protein than casein.

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[3] Supplemental Tables 1–8, Supplemental Figure 1, and Supplemental Methods are available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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Human studies have evaluated the impact of dietary supplementation with soy protein on fecal microbial changes and how these changes affect the gut metabolism of the associated isoflavones (9–11). Fernandez-Raudales et al. (12) reported that obese adult men consuming a low glycinin or conventional soymilk for 3 mo had significantly lower *Firmicutes*-to-*Bacteroidetes* ratios and lower *Bifidobacterium* than a bovine milk-fed group.

Soy protein consumption is associated with several cardiometabolic benefits including reduced plasma lipids (13, 14); however, the exact mechanisms whereby soy protein reduces plasma lipids are unknown (15). Dietary components that lower serum cholesterol concentrations have been shown to alter gut microbiota in hamsters, a model of human lipid metabolism (16, 17). It is conceivable that soy protein may also induce similar changes in the gut microbiota.

We hypothesized that consumption of soy protein rather than animal-derived protein can differentially modulate the gut microbiome, and this may account for the beneficial effects of soy protein on lipid metabolism. The objective of the current study was to investigate the effects of different protein sources on the gut microbiota composition in hamsters and determine whether these changes could account for any of the lipid-lowering activity of soy protein. In the current study, diets mimicking the composition of a typical Westernized human diet containing either milk protein isolate (MPI)[4] or 1 of 3 differently processed soy proteins were investigated for their effects on serum lipids, microbiota composition in the gut, and expression of genes involved in hepatic lipid metabolism. A secondary objective was to determine how differences in processing of soy protein may modulate its ability to alter serum lipids and gut microbiota composition.

Methods

Animals and diets. Six- to 8-wk-old male Golden Syrian hamsters (Charles River Laboratories, Kingston, New York) were housed individually in cages with wire bottom inserts and maintained in a temperature-controlled room, with a 12-h light/dark cycle, and allowed free access to food and water. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the Department of Comparative Medicine, St. Louis University, and conducted by Seventh Wave Laboratories. Animals were cared for according to the NIH Guide for the Care and Use of Laboratory Animals. Semipurified diets were prepared by Research Diets, Inc., New Brunswick, New Jersey, and are described in **Supplemental Table 1**. All hamsters were fed a semipurified diet containing 22% (%wt) MPI ad libitum for 3 wk. After this acclimation period, the animals were randomly assigned by serpentine sorting, based on total cholesterol concentrations, to 1 of 4 groups (n = 8/group) for an additional 6 wk [22% (%wt) protein]: MPI, soy protein concentrate (SPC), partially hydrolyzed soy protein isolate (SPI1), or intact soy protein isolate 2 (SPI2).

Sample collection and analysis of cardiometabolic markers. Body weights and food consumption were monitored and recorded weekly starting at day 0 of test diet initiation. Spillage and leftover food were recorded weekly to determine food consumption. Feces were collected during the first 3 h of the dark period on the last day of the acclimation period (day −1) and then on days 6, 21, and 41 following test diet initiation, snap-frozen, and stored immediately at −80°C. At study termination, hamsters were anesthetized by CO_2 and killed by exsanguination. Right gastrocnemius muscle, epididymal fat pads, and liver were immediately excised, recorded for weight, and snap-frozen in liquid nitrogen. Tissue samples were also collected from the small intestine (duodenum, jejunum, and ileum) and lower intestine (cecum and colon), and cecal contents. The tissue samples were equalized in weight for each intestinal collection site. The luminal contents were gently removed from the lower intestinal tissues with the use of sterile buffered peptone water (Hardy Diagnostics, Santa Maria, California). Each tissue sample was homogenized (Masticator; IUL Instruments, Barcelona, Spain) for 60 s with a 1:10 dilution of buffered peptone water. Tissue homogenates and undiluted cecal contents were snap-frozen in liquid nitrogen and stored at −80°C until subsequent microbial analysis. Terminal, nonfasting, heparinized blood samples were collected by vena cava stick and frozen at −80°C to measure glycated hemoglobin as an indicator of average blood glucose concentrations (Diabetes Diagnostic Labs, University of Missouri, Columbia, Missouri). Nonfasting serum was isolated from the terminal blood collection by centrifugation at 1600 × g for 10 min and frozen at −80°C. Individual lipoprotein cholesterol profiles and total TG concentrations were analyzed by means of the vertical auto profile method (Atherotech Diagnostics Lab, Birmingham, Alabama) (18).

Characterization of fecal and intestinal microbiota. DNA was isolated from the fecal and intestinal samples with the use of the PowerMag Soil DNA Isolation Kit (MO-BIO Laboratories, Carlsbad, California) by means of the KingFisher96 purification system (Fisher Scientific, Waltham, Massachusetts) with the addition of a 10-min lysis incubation at 70°C. The microbial community composition was analyzed with the use of high throughput amplicon sequencing as previously described (19). Briefly, the V4 variable region of the 16S ribosomal RNA gene of bacteria and archaea was amplified by use of primers 515F (5′-GTGCCAGCMGCCGCGGTAA) and 806R (5′-GGACTACHVGGGTWTCTAAT) with the addition of appropriate Illumina sequencing adapters and a unique 12-bp Golay barcode in the reverse primer. Amplicon libraries were prepared and sequenced on the Illumina MiSeq platform (Argonne National Laboratory, IGSB-NGS Core Facility, Lemont, Illinois). The sequencing data were processed and analyzed by use of the Quantitative Insights into Microbial Ecology (v. 1.8) pipeline (20). Refer to **Supplemental Methods** for quality filtering and sequence processing. A nonparametric t test using 1000 Monte Carlo permutations and Benjamini-Hochberg false discovery rate correction was used to make α-diversity (within-sample richness) comparisons. The β-diversity (between-sample dissimilarity) estimates were calculated by means of UniFrac metrics (21, 22) on rarefied OTU tables and visualized with the use of principal coordinate analysis. Group differences were assessed by means of permutational multivariate analysis of variation based on the UniFrac distance matrix in Quantitative Insights into Microbial Ecology.

Gene expression in liver. Reference and primer sequences for target genes analyzed by qPCR can be found in **Supplemental Table 2** with the accompanying methods for hepatic gene expression.

Statistics. All cardiometabolic markers and gene expression data were analyzed separately by means of a 1-factor ANOVA with group as the main effect. Post hoc Tukey's honestly significant difference test (HSD) pairwise comparisons were conducted to identify statistical differences between the groups when a significant main effect was found. Discriminate microbes were identified for summarized taxa and OTUs present at a minimum of 0.1% abundance threshold. Nonparametric Kruskal-Wallis tests with the Benjamini-Hochberg false discovery rate correction were conducted with a main effect of the group for each taxa and OTU. Multiple comparisons between the groups were made by using Steel-Dwass comparisons. For fecal samples, time points (days −1, 6, 21, and 41) were analyzed separately. Family level data are reported in the Results section, and the remainder of the data are reported in **Supplemental Table 3**. To increase power, additional analyses were conducted on cecal microbes, comparing the 3 soy-fed groups combined to the MPI group by means of nonparametric Wilcoxon's rank-sum test. Two-way cluster analysis was performed on family level cecal microbes (relative abundance) z scores and group means. The z scores were

[4] Abbreviations used: MPI, milk protein isolate; OTU, operational taxonomic unit; SPC, soy protein concentrate; SPI1, soy protein isolate 1; SPI2, soy protein isolate 2.

TABLE 1 Food consumption, body weights, and tissue weights of hamsters fed soy or milk protein for 6 wk[1]

	Dietary group				ANOVA
	MPI	SPC	SPI1	SPI2	*P* value
Food intake,[2] g/d	7.6 ± 0.26[b]	8.4 ± 0.50[a,b]	9.0 ± 0.39[a]	7.6 ± 0.25[b]	0.021
Body weight, g					
Baseline, day 0	109 ± 4.10	107 ± 2.91	107 ± 2.78	106 ± 2.62	0.93
Terminal, day 42	116 ± 3.49	116 ± 2.96	124 ± 3.55	118 ± 3.08	0.29
Epididymal fat/body weight, mg/g	9.5 ± 0.71	10.0 ± 0.71	10.5 ± 0.68	9.8 ± 0.98	0.83
Gastrocnemius/body weight, mg/g	2.2 ± 0.08	2.1 ± 0.05	2.2 ± 0.09	2.2 ± 0.03	0.94
Liver/body weight, g/g	0.037 ± 0.001	0.037 ± 0.001	0.039 ± 0.001	0.037 ± 0.001	0.43

[1] Values are means ± SEMs, $n = 8$. Labeled means in a row without a common superscript letter differ, $P < 0.05$ [Tukey's honestly significant difference test (HSD)]. MPI, milk protein isolate; SPC, soy protein concentrate; SPI1, soy protein isolate 1; SPI2, soy protein isolate 2.
[2] Food intake is the average intake/d over 41 d.

calculated for each microbial family by subtracting the mean and dividing by the SD. Group means were used in the cluster analysis to simplify the heatmap. Hierarchical cluster analysis with Ward's minimum variance method was selected. Data were standardized robustly with the use of Huber M-estimates, which reduces the influence of outliers and produces a heatmap with uniform scaling across groups. To determine relation among the different outcome measures, Spearman correlation analysis was conducted for microbial cecal families, cardiometabolic markers, tissue weights normalized to body weights, body weights, and gene expression data, for all groups together. The α was set to 0.05 for all statistical analyses, and $P \leq 0.05$ was considered significant. Before parametric analyses compared the treatment groups, the Brown-Forsythe test of homogeneity of variances was conducted. The majority of variances were observed to be equal among the treatment groups for the cardiometabolic and genetic outcomes. When the Brown-Forsythe test was found to be significant ($P < 0.05$), indicating unequal variances, a Welch test was conducted. Results of the Welch tests were found to be comparable to the ANOVA *F* tests. Therefore, the results of the ANOVA tests are reported. All results are expressed as means ± SEMs unless indicated. All analyses were performed with JMP Software v. 10 (Cary, North Carolina).

Results

Effects of dietary protein on food consumption, body weight, tissue weights, and cardiometabolic markers. The SPI1 group consumed significantly more food than the MPI group and SPI2-fed groups ($P < 0.05$), but there were no significant group effects for baseline and terminal body weights or tissue weights (**Table 1**). Total cholesterol concentrations were significantly lower in the SPC group than the MPI-fed group and SPI2-fed group ($P < 0.05$) (**Table 2**). Serum TGs were lower in the SPI1-fed group than the MPI-fed group ($P < 0.05$), and LDL cholesterol was lower in the SPC group than the MPI-, SPI1-, and SPI2-fed groups ($P < 0.05$) (Table 2). In addition, VLDL cholesterol, $VLDL_{1+2}$ cholesterol, and non-HDL cholesterol were significantly lower for each soy-fed group than for the MPI-fed group ($P < 0.05$ for all) (Table 2). Non-HDL cholesterol in SPC-fed hamsters was lower than that in the SPI1- and SPI2-fed groups ($P < 0.05$) (Table 2). HDL-cholesterol concentrations did not differ between the groups ($P = 0.18$). Serum apoA-I concentrations tended to be lower in the SPC group than in the MPI-fed animals ($P = 0.06$); however, the apoB concentration and apoB-to-apoA-I ratio did not differ between the groups ($P = 0.28$) (Table 2).

Effects of dietary protein on intestinal microbiota. α-Diversity (within-sample) indexes indicated that overall the soy-fed hamsters contained a more diverse microbiota than those fed the MPI diet, as characterized by a greater number of observed OTUs in both

TABLE 2 Whole blood and serum cardiometabolic measures of hamsters fed soy or milk protein for 6 wk[1]

	Dietary group				ANOVA
	MPI	SPC	SPI1	SPI2	*P* value
HbA1c, %	4.78 ± 0.08	4.60 ± 0.07	4.71 ± 0.08	4.63 ± 0.08	0.37
Total cholesterol, mg/dL	238 ± 8.69[a]	183 ± 9.04[b]	210 ± 5.34[a,b]	215 ± 8.65[a]	0.0007
HDL-C, mg/dL	131 ± 6.21	115 ± 4.88	126 ± 4.55	127 ± 5.31	0.18
Non-HDL-C, mg/dL	107 ± 4.17[a]	67.9 ± 4.34[c]	84.5 ± 3.26[b]	88.3 ± 3.57[b]	<0.0001
LDL-C, mg/dL	72 ± 3.9[a]	50 ± 4.2[b]	66 ± 2.4[a]	67 ± 4.1[a]	0.002
VLDL-C, mg/dL	35 ± 1.9[a]	18 ± 0.82[b]	19 ± 1.9[b]	21 ± 1.7[b]	<0.0001
$VLDL_{1+2}$-C, mg/dL	17 ± 1.3[a]	7.5 ± 0.52[b]	7.2 ± 0.90[b]	8.9 ± 1.2[b]	<0.0001
TG, mg/dL	223 ± 14.2[a]	171 ± 20.7[a,b]	140 ± 20.8[b]	155 ± 22.3[a,b]	0.046
apoB, mg/dL	64 ± 3.0	50 ± 2.6	57 ± 3.0	62 ± 5.2	0.045
apoA-I, mg/dL	280 ± 8.74	249 ± 6.89	260 ± 7.67	265 ± 7.07	0.06
apoB/apoA-I	0.23 ± 0.01	0.20 ± 0.01	0.22 ± 0.02	0.24 ± 0.01	0.28

[1] Values are means ± SEMs, $n = 8$. Labeled means in a row without a common superscript letter differ, $P < 0.05$ [Tukey's honestly significant difference test (HSD)]. HbA1c, glycated hemoglobin; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; MPI, milk protein isolate; SPC, soy protein concentrate; SPI1, soy protein isolate 1; SPI2, soy protein isolate 2; VLDL-C, VLDL cholesterol; $VLDL_{1+2}$-C, VLDL subfraction 1 and 2 cholesterol.

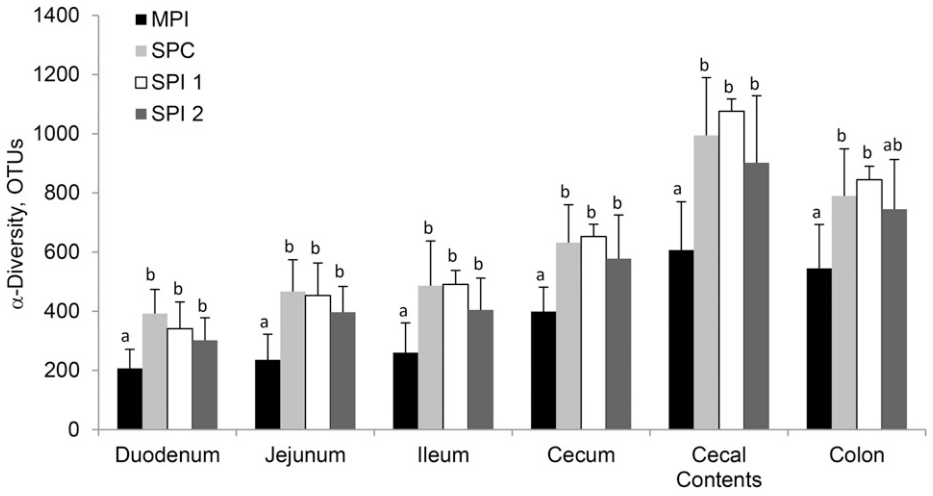

FIGURE 1 The α-diversity averaged by intestinal section of hamsters fed soy or milk protein for 6 wk. Observed OTUs represent clusters at 97% sequence similarity. Values are means ± SDs, n = 7–8. Labeled means without a common letter differ, $P \leq 0.05$ (nonparametric t test using 1000 Monte Carlo permutations and Benjamini-Hochberg False Discovery Rate correction). MPI, milk protein isolate; OTU, operational taxonomic unit; SPC, soy protein concentrate; SPI 1, soy protein isolate 1; SPI 2, soy protein isolate 2.

the small and large intestinal samples (**Figure 1**). β-Diversity (between-sample dissimilarity) based on the weighted UniFrac distance of hamster microbial communities in the cecum revealed distinct groupings for the MPI-fed rather than the soy-fed groups and significant sample clustering by diet (all soy-fed groups pooled for statistical analyses compared with MPI; permutational multivariate analysis of variation, P = 0.001) (**Figure 2**).

Dietary protein source had a significant impact on the composition of the gut microbiota. Microbial differences were observed among the groups at all taxonomic levels and at every intestinal site sampled. The small intestine was dominated by *Firmicutes* and *Bacteroidetes* (>90% of the total abundance; data not shown). In the duodenum, only phylum level differences ($P \leq 0.05$) were detected among the groups, in which there was a lower abundance of *Bacteriodetes* and a higher abundance of *Proteobacteria* in the MPI-fed group (Supplemental Table 3). Microbial shifts in the ileum, and to a lesser degree in the jejunum, were observed at finer taxonomic levels. Specifically, the family Erysipelotrichaceae, which entirely comprised *Allobaculum* spp., was highly abundant (60% of the total abundance) in the ileum of MPI-fed hamsters compared with 30–35% of the total in the soy-fed groups. *Coriobacteriaceae* containing the genus *Adlercreutzia* was also higher in the ileum of the MPI-fed group (Supplemental Table 3).

Before the start of the study, we determined that the intestinal region of highest interest was the cecum, as it is the site of microbial fermentation and harbors a high concentration of diverse microbes. The cecal microbiota was dominated by *Firmicutes* (47%) and *Bacteriodetes* (42%), with the predominant families being *S24–7* (41%), *Erysipelotrichaceae* (16%), *Clostridiales* spp. (13%), *Ruminococcaceae* (8%), and *Bacteroidaceae* (4%) across all groups (data not shown).

Multiple comparisons were made between groups at the taxonomic family level in the different intestinal sections (**Table 3**). In the lower intestine, the largest microbial shifts were found within the Bacteroidetes phylum, with a higher relative abundance of *Bacteroidaceae* and *Porphyromonadaceae* in the MPI-fed group and higher relative abundance of *S24–7* in the soy-fed groups. Cluster analysis (z scores) determined that the soy-fed groups were more similar to one another, because they clustered together first, whereas the MPI-fed group was in a cluster of its own (**Supplemental Figure 1**). The SPI1-fed group, however, appeared to have some unique effects on the intestinal microbiota. Specifically, there were elevated levels of *Bifidobacteriaceae* in the SPI1-fed group and a higher proportion of *Clostridales* spp. as well as significant differences in several low abundant taxa compared with the MPI-fed group (Table 3).

Fecal samples were also analyzed for their microbial composition to investigate the effect of diet over time. Relative abundance of the microbial families at different time points showing the greatest differences between groups is depicted in **Figure 3** and **Supplemental Table 4**. Only significant differences ($P \leq 0.05$) among families present at >1% of the total abundance of fecal microbiota are reported. Significant differences were found among groups for *S24–7* at day 21 and day 41 ($P < 0.05$ and $P = 0.01$, respectively), and *Clostridiales* spp. also showed significant differences among groups at day 21 and day 41 ($P < 0.01$ and $P < 0.05$, respectively). The MPI-fed group

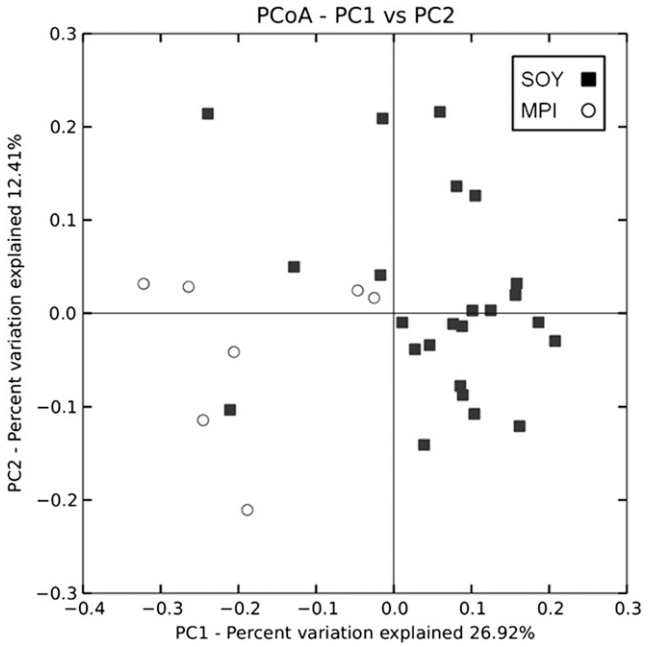

FIGURE 2 PCoA based on weighted UniFrac distances of microbial communities in the cecum of hamsters fed soy or milk protein for 6 wk. Sample clustering is significant (P = 0.001, PERMANOVA) for MPI (circles) compared with SOY (squares). MPI (n = 7); SOY (n = 24). MPI, milk protein isolate; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variation; SOY, all soy-fed groups.

TABLE 3 Family level relative abundance of microbes differing among dietary groups in intestinal samples of hamsters fed soy or milk protein for 6 wk[1]

Intestinal section and microbial family	Dietary group, % abundance				*P* value
	MPI	SPC	SPI1	SPI2	
Ileum					
Bifidobacteriaceae	0.41 ± 0.10[b]	0.91 ± 0.20[a,b]	3.4 ± 1.3[a]	1.3 ± 0.8[a,b]	0.05
Coriobacteriaceae	0.91 ± 0.39[a]	0.11 ± 0.02[b]	0.12 ± 0.03[b]	0.22 ± 0.09[a,b]	0.05
Erysipelotrichaceae	59 ± 5.3[a]	34 ± 6.2[a,b]	31 ± 3.4[b]	35 ± 5.1[b]	0.05
Cecum					
Alcaligenaceae	1.3 ± 0.86[a]	0.94 ± 0.65[a,b]	0.53 ± 0.03[b]	0.41 ± 0.29[a,b]	0.03
Bacteroidaceae	14 ± 45[a]	0.73 ± 0.40[b]	0.24 ± 0.10[b]	1.5 ± 0.74[b]	0.03
Bifidobacteriaceae	0.08 ± 0.04[b]	0.28 ± 0.08[a,b]	2.5 ± 0.87[a]	0.58 ± 0.30[a,b]	0.03
Clostridiales spp.	7.8 ± 1.5[b]	11 ± 1.9[a,b]	18 ± 2.5[a]	13 ± 1.4[a,b]	0.05
Deferribacteraceae	0.12 ± 0.12[b]	0.77 ± 0.22[a,b]	0.82 ± 0.37[a]	0.48 ± 0.28[a,b]	0.05
Helicobacteraceae	0.78 ± 0.42[a]	1.4 ± 0.35[a,b]	0.11 ± 0.05[b]	0.24 ± 0.09[a,b]	0.05
Porphyromonadaceae	2.5 ± 1.0[a]	0.37 ± 0.12[a,b]	0.13 ± 0.06[b]	0.35 ± 0.16[a,b]	0.05
S24–7	27 ± 5.7[b]	50 ± 2.6[a]	42 ± 3.3[a,b]	43 ± 5.7[a,b]	0.05
Cecal contents					
Bacteroidaceae	13 ± 4.5[a]	1.4 ± 1.1[b]	0.38 ± 0.09[b]	1.7 ± 0.87[b]	0.02
Bifidobacteriaceae	0.06 ± 0.03[b]	0.28 ± 0.08[a]	1.7 ± 0.65[a]	0.41 ± 0.22[a,b]	0.02
Deferribacteraceae	0.04 ± 0.03[b]	0.15 ± 0.07[a,b]	0.31 ± 0.10[a]	0.14 ± 0.08[a,b]	0.02
Lachnospiraceae	2.7 ± 0.53[b]	9.2 ± 2.1[a]	17 ± 2.9[a,b]	7.5 ± 1.7[a,b]	0.05
Porphyromonadaceae	2.5 ± 0.88[a]	0.71 ± 0.40[b]	0.24 ± 0.08[b]	0.83 ± 0.45[b]	0.03
RF39 spp.	0.01 ± 0.01[b]	0.41 ± 0.16[a]	0.12 ± 0.05[a,b]	0.08 ± 0.04[a,b]	0.04
Colon					
Bacteroidaceae	14 ± 4.3[a]	1.1 ± 0.76[b]	0.23 ± 0.07[b]	1.5 ± 0.72[b]	0.02
Porphyromonadaceae	2.3 ± 0.86[a]	0.42 ± 0.22[b]	0.14 ± 0.05[b]	0.48 ± 0.19[a,b]	0.02

[1] Values are means ± SEMs, $n = 7–8$. Nonparametric Kruskal-Wallis tests with the Benjamini-Hochberg false discovery rate correction were conducted with a main effect of group. Labeled means in a row without a common superscript letter differ, $P \leq 0.05$ (Steel-Dwass). MPI, milk protein isolate; SPC, soy protein concentrate; SPI1, soy protein isolate 1; SPI2, soy protein isolate 2.

showed a relative increase in *Erysipelotrichaceae* than did all soy-fed groups; this was significant only at day 41 ($P < 0.01$). *Bacteroidaceae* was elevated in the MPI-fed group; however, differences were only significant at day 21 ($P = 0.01$).

Effects of dietary protein on gene expression in the liver. When compared with that of MPI, intake of soy proteins resulted in significant upregulation of multiple genes involved in cholesterol synthesis including *Fdft* ($P < 0.01$), *Hmgcr* ($P < 0.001$), and *Lss* ($P < 0.0001$) (**Table 4**). *Hsd17b7* expression was significantly higher in hamsters fed SPC ($P < 0.05$) and SPI1 ($P < 0.05$), but not SPI2 compared with MPI. *Scd1* ($P < 0.01$) mRNA levels were lower by ~50% in each of the 3 soy-fed groups. *Me1* mRNA levels were lower with SPI1 consumption only when compared with those of SPC ($P < 0.05$); *Me1* gene expression was not significantly different in SPI1-fed compared with MPI- or SPI2-fed hamsters. *Wnt10b* was downregulated in only the SPI1 group ($P < 0.05$ compared with the MPI-fed group). No significant differences were observed between groups in the expression of *apoA-I*, *Fads1*, *Fasn*, *Hmgcs2*, *Nfe2l1* and *Nfe2l2*, *Lrp6*, *Pcsk9,* and *Wnt5a*.

Correlation of cecal microbes with lipid parameters. Spearman correlations of microbial families in the cecum with lipid parameters can be found in **Supplemental Table 5**. When data for all soy-fed groups were pooled for analyses, 4 microbial families were present at significantly higher abundance in the cecum of all the soy-fed groups than in the MPI-fed group, namely *S24–7*, *Bifidobacteriaceae*, *Clostridiales* spp., and *Deferribacteraceae* ($P < 0.05$). In contrast, 3 microbial families were present in significantly higher abundance in the MPI-fed group than in all the soy-fed groups (*Alcaligenaceae*, *Bacteroidaceae,* and *Porphyromonoadaceae*) ($P < 0.05$). Serum total LDL-cholesterol, $VLDL_{1+2}$-cholesterol, and non-HDL-cholesterol concentrations were inversely correlated with the percent abundance of *S24–7* and *Deferribacteraceae*, which were more prevalent in the soy-fed groups. $VLDL_{1+2}$-cholesterol concentrations were also inversely correlated with *Bifidobacteriaceae*, which were more prevalent in the soy-fed groups. In contrast, $VLDL_{1+2}$ cholesterol, non-HDL cholesterol, and TG concentrations were positively correlated with *Bacteroidaceae*, which were present in higher abundance in the MPI-fed group. Also, $VLDL_{1+2}$-cholesterol concentrations were positively correlated with *Pophyromonoadaceae*, a family also more prevalent in the MPI-fed group than in the soy-fed groups.

Correlation of cecal microbes with hepatic gene expression. Serum cholesterol concentrations were inversely correlated with the expression of several genes in the cholesterol biosynthetic pathway in the liver (*Fdft1*, *Hmgcr*, *Hsd17b7*, and *Lss*; data not shown), consistent with feedback inhibition due to reduced serum cholesterol concentrations. To determine whether there was a relation between hepatic gene expression and the abundance of cecal microbial families, Spearman correlation analysis was conducted and those that were found to be significant between at least 1 microbial family and hepatic genes involved in lipid metabolism are shown in **Supplemental Table 6**. Positive correlations of cholesterol biosynthetic gene expression and the abundance of microbial families found to be more abundant in the soy-fed groups were observed (e.g.,

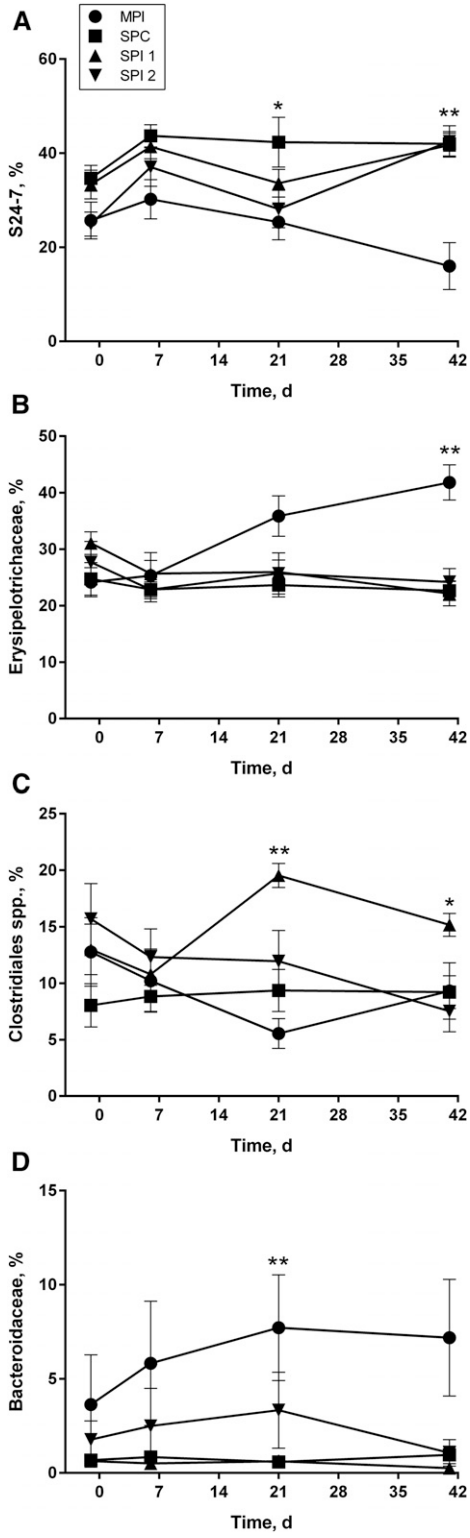

FIGURE 3 Fecal microbial families (>1% of the total abundance) of hamsters fed soy or milk protein for 6 wk: $n = 32$ (day −1, day 6, and day 21) or $n = 29$ (day 41). Values are means ± SEMs. For each time point $^*P \leq 0.05$, $^{**}P \leq 0.01$, group effect using Kruskal-Wallis. Multiple comparisons between groups are reported in Supplemental Table 4. MPI, milk protein isolate; SPC, soy protein concentrate; SPI 1, soy protein isolate 1; SPI 2, soy protein isolate 2.

Bifidobacteriaceae abundance and *Hmgcr* expression, $\rho = 0.62$, $P = 0.0002$); in contrast, there was an inverse correlation between microbial families in higher abundance in the MPI-fed group and cholesterol biosynthetic gene expression (e.g., *Bacteroidaceae* abundance and *Hmgcr* expression, $\rho = -0.57$, $P = 0.001$). Expression of FA metabolism–associated genes, *Fasn*, *Scd1,* and *Me1*, tended to be inversely correlated with the abundance of microbial families in the soy-fed groups and was positively correlated with the abundance of microbial families present in the MPI-fed group (e.g., *Clostridiales* spp. abundance and *Scd1* expression in all soy-fed groups, $\rho = -0.55$, $P = 0.002$; and *Bacteroidaceae* abundance and *Scd1* expression in MPI-fed group, $\rho = 0.57$, $P = 0.001$). Hepatic *apoA-I* gene expression tended to be inversely correlated with microbial families more abundant in the soy-fed groups and more positively correlated with those prevalent in the MPI-fed group. Inverse correlations were also observed between *Lrp6* (a *Wnt* co-receptor), *Wnt10b*, and *Wnt5a* ligands and microbial families with individual microbial families that were more abundant in the soy-fed groups. In contrast, *Lrp6* and *Wnt10b* tended to be positively correlated with microbial families more abundant in the MPI-fed group.

Correlation of Wnt5a *gene expression and unclassified Clostridiales with body weight.* In analyses of Spearman correlations between microbial families, hepatic gene expression, cardiometabolic markers, and anthropometric measures, we noted a consistent inverse correlation in all diet groups between *Wnt5a* expression and body weights taken at and after day 13 of the study (terminal $\rho = -0.49$, $P = 0.016$) (**Supplemental Table 7**). Body weights and the presence of unclassified *Clostridiales* in the cecum were also positively correlated after day 34. No other significant correlations with microbial families and body weight were observed.

Correlations of terminal muscle weight and HDL cholesterol biomarkers. Terminal muscle weights in all diet groups were found to correlate significantly with serum apoA-I, HDL-cholesterol, and HDL_2-cholesterol concentrations (**Supplemental Table 8**).

Discussion

The major findings of this study are *1*) consumption of soy protein rather than milk protein diets by hamsters resulted in significant reductions in the concentrations of serum total cholesterol, TGs, and atherogenic lipoprotein particles; *2*) gut microbiota profiles from all soy-fed groups were more similar to each other and showed significant differences in abundance of several key microbial families than the microbiota profiles from the MPI-fed group; and *3*) correlation analyses revealed associations of the gut microbiota profiles with serum lipid concentrations and hepatic gene expression, suggesting that some of the lipid-lowering activity of soy protein may be due to changes in gut microbial profiles. Reductions in plasma lipids in the soy-fed groups were not due to changes in body weight or food intake because these were similar or even higher than what was observed in the MPI-fed group (Table 1). The reason for the variability in serum lipid responses in response to the differently processed soy protein ingredients (Table 2) is not known and may be due to subtle processing differences, since the soy proteins are virtually identical in overall amino acid composition (see example composition and comparison to milk protein in reference 15). SPC is a water-based protein concentrate produced with membrane technology, whereas both SPI1 and SPI2 are isolates that were generated by isoelectric

TABLE 4 Hepatic expression of genes associated with lipid metabolism and *Wnt* pathway signaling of hamsters fed soy or milk protein for 6 wk[1]

Gene	Gene product (protein)	Relative gene expression by dietary group, fold of MPI				ANOVA
		MPI	SPC	SPI1	SPI2	*P* value
apoA-I	apolipoprotein A-I	1.0 ± 0.07	0.92 ± 0.05	0.81 ± 0.05	0.96 ± 0.07	0.12
Fads1	FA desaturase 1	1.1 ± 0.11	1.2 ± 0.09	1.1 ± 0.08	1.4 ± 0.13	0.24
Fasn	FA synthase	1.1 ± 0.13	1.0 ± 0.07	0.80 ± 0.08	0.86 ± 0.06	0.069
Fdft1	Farnesyl-diphosphosphate farnesyl-transferase 1	1.1 ± 0.12^{b}	1.9 ± 0.17^{a}	2.1 ± 0.14^{a}	1.7 ± 0.17^{a}	<0.001
Hmgcr	3-Hydroxy-3-methylglutaryl-CoA reductase	1.1 ± 0.13^{b}	2.5 ± 0.37^{a}	2.7 ± 0.25^{a}	2.1 ± 0.15^{a}	<0.001
Hmgcs2	3-Hydroxy-3-methylglutaryl-CoA synthase 2	1.0 ± 0.07	0.95 ± 0.07	0.95 ± 0.07	1.1 ± 0.12	0.80
Hsd17b7	17-β-Hydroxysteroid dehydrogenase 7	1.1 ± 0.13^{b}	1.8 ± 0.22^{a}	1.7 ± 0.13^{a}	1.5 ± 0.16^{a,b}	0.012
Nfe2l1	Nuclear factor, erythroid 2-like 1	1.0 ± 0.07	0.96 ± 0.06	0.96 ± 0.06	1.0 ± 0.09	0.85
Nfe2l2	Nuclear factor, erythroid 2-like 2	1.0 ± 0.04	0.90 ± 0.06	0.95 ± 0.07	0.97 ± 0.10	0.42
Lrp6	LDL-related protein 6	1.0 ± 0.06	0.88 ± 0.04	0.86 ± 0.06	0.95 ± 0.08	0.18
Lss	Lanosterol synthase	1.1 ± 0.08^{b}	2.1 ± 0.16^{a}	2.1 ± 0.09^{a}	1.9 ± 0.16^{a}	<0.001
Me1	Malic enzyme 1, NADP(±)-dependent, cytosolic	1.1 ± 0.11^{a,b}	1.3 ± 0.14^{a}	0.79 ± 0.10^{b}	0.94 ± 0.14^{a,b}	0.044
Pcsk9	Proprotein convertase subtilisin/kexin type 9	1.1 ± 0.11	1.3 ± 0.17	1.4 ± 0.07	1.5 ± 0.11	0.16
Scd1	Steroyl-CoA desaturase-1	1.0 ± 0.08^{a}	0.60 ± 0.06^{b}	0.54 ± 0.07^{b}	0.63 ± 0.08^{b}	<0.001
Wnt10b	Wingless-type MMTV integration site 10b	1.5 ± 0.41^{a}	1.2 ± 0.23^{a,b}	0.53 ± 0.10^{b}	0.71 ± 0.11^{a,b}	0.014
Wnt5a	Wingless-type MMTV integration site 5a	1.2 ± 0.17	0.99 ± 0.13	0.86 ± 0.23	0.60 ± 0.08	0.06

[1] Values are mean ratios ± SEMs, $n = 7$–8, using the MPI group as the calibrator control and *ACTB* gene expression as the normalizing reference control. Labeled means in a row without a common superscript letter differ, $P < 0.05$ [Tukey's honestly significant difference test (HSD)]. *ACTB*, β-actin; MMTV, mouse mammary tumor virus; MPI, milk protein isolate; NADP, nicotinamide adenine dinucleotide phosphate; SPC, soy protein concentrate; SPI1, soy protein isolate 1; SPI2, soy protein isolate 2; *Wnt*, wingless-related integration site.

precipitation of the soy protein. SPI1 differs from SPI2 in that it is also partially hydrolyzed by limited food enzyme treatment. These processing differences may affect the micronutrient content and/or overall disposition of the protein components in vivo. Nonetheless, all soy proteins resulted in significant reductions in non-HDL cholesterol and $VLDL_{1+2}$ cholesterol than in the MPI-fed group (Table 2). Non-HDL cholesterol is considered to be a more predictive risk factor for cardiovascular disease than LDL cholesterol (23). The larger reduction in $VLDL_{1+2}$ cholesterol than in LDL cholesterol is consistent with the findings of Oliva et al. (24) and Tovar and Torres (25) that soy protein consumption reduces VLDL secretion rates in animal models.

The soy-fed hamsters had consistently more diverse microbiota in the small and lower intestine than the MPI-fed group. Increased microbial diversity in the gut microbiome has been associated with a "lean" phenotype (26). It has also been suggested that microbial richness is a marker of metabolic health, in which individuals with low richness have a higher incidence of dyslipidemia, adiposity weight gain, insulin resistance, and inflammation (27).

Alterations in the microbial composition between the soy- and the MPI-fed groups were observed at all taxonomic levels and all intestinal sites sampled (Supplemental Table 3, Table 3). The largest differences were found within the *Bacteroidetes* phylum, where the MPI-fed group had a higher relative abundance of *Bacteroidaceae* and *Porphyromonadaceae* than the soy-fed groups, which were dominated by *S24–7* (a prevalent member of the rodent, but not the human, intestinal microbiota). SPI1-fed hamsters were unique in exhibiting a bloom of *Bifidobacteriaceae* (across most intestinal sections) and a higher proportion of *Clostriadiales* spp. in the cecum than did the MPI-fed group (Table 3). Bifidobacteria are lactic acid bacteria commonly used as probiotics for human health and have been shown to reduce host cholesterol concentrations or ameliorate risk factors for metabolic syndrome (28–32). SPC and SPI2 also lowered serum lipids but did not exhibit a marked increase in *Bifidobacteriaceae*, suggesting that alterations in this microbial family alone do not account for the lipid-lowering effects of soy protein. The MPI-fed group was characterized by a greater abundance of *Erysipelotrichaceae* in ileum and fecal samples (Table 3, Figure 3), and studies in hamsters (16) and humans (33) have associated *Erysipelotrichaceae* with dyslipidemic phenotypes. *Erysipelotrichaceae* abundance has been previously shown to increase with cholesterol-enriched diets in rats and decrease with the addition of soy protein (7). Changes in the fecal microbial profiles over time occurred predominantly in the MPI-fed group, despite being on the same diet during the run-in period of the study. This cannot be explained (Figure 3) by any treatment differences in soy-fed compared with MPI-fed hamsters. Changes in the microbial profile of the MPI-fed group may have resulted from modulation of biochemical signals between the liver and intestine over time (34).

Lipid concentrations were inversely correlated with microbial families that were more abundant in soy-fed rather than the MPI-fed group (Supplemental Table 5). Differences in the microbial profiles between soy-fed and MPI-fed groups may be due to differences in the way the protein components affect gut luminal contents and/or gut function, either indirectly by affecting bile acid metabolism (15, 35, 36) or directly through antimicrobial activities of dietary protein–derived peptides (37–39).

Although not necessarily defining a cause and effect, the observed correlations between the abundance of specific microbial families and concentrations of serum lipids in the soy-fed compared with the MPI-fed hamsters infer an association between these microbial families and hamster lipid metabolism. Claus et al. (40) demonstrated an association between the abundance of a specific gut microbial family and murine lipid metabolism. Microbial-derived propionate and bile acid metabolites have been linked to direct effects on host hepatic gene expression and lipogenesis (41, 42). In this study, expression of genes in the cholesterol biosynthetic, FA metabolism, and *Wnt* signaling pathways was different between soy-fed and MPI-fed

hamsters. Upregulation of *Hmgcr*, *Fdft1*, *Hsd17b7*, and *Lss* expression in the soy-fed rather than the MPI-fed hamsters was likely due to a feedback response to coincident reductions in serum cholesterol concentrations, observed previously in rats fed soy protein (43) or prebiotic fiber (44). The soy protein–mediated decrease in serum cholesterol concentrations may be secondary to reduced VLDL production and secretion. Specifically, *Scd1* expression, which stimulates lipogenesis and VLDL production (45), was significantly lowered in the soy-fed groups rather than the MPI-fed group (Table 4), consistent with observations in other animal studies (46–48). Gene expression of *Me1*, an enzyme that plays a major role in lipogenesis (49), was significantly lower in the SPI1-fed hamsters than in the MPI-fed animals (Table 4), again consistent with previous studies in rats and mice (43, 50). In the current study, SPI1- and SPI2-fed groups showed a trend but no significant reduction of *Fasn* expression compared with MPI-fed group. Overall, consumption of soy protein was associated with lower expression of genes in hepatic lipogenic pathways than consumption of MPI, which may also explain the reduction of fatty liver by soy rather than by dairy protein consumption observed in other animal studies (24, 43, 46, 48, 51, 52).

Correlations between cecal microbial families and expression of specific genes diverged by whether the families were more abundant in the soy-fed groups than the MPI-fed group (Supplemental Table 6), suggesting that gut signals may modulate hepatic cholesterol gene expression. The expression of prolipogenic *Scd-1* was inversely correlated with the abundance of *Bifidobacteriaceae* and *Clostridiales* spp., which were more prevalent in the soy-fed groups, whereas there was a significant positive correlation of *Scd-1* expression with microbial families that were more abundant in the MPI-fed group. *Alcaligenaceae* and *Porphyromonadaceae*, which were more abundant in the MPI-fed group, were also positively correlated with the expression of *Me1*. The significant correlations that were identified between microbial families and lipogenic gene expression are consistent with the concept of a direct association between gut microbiota and host hepatic lipid metabolism (40).

An unexpected inverse correlation between *Wnt5a* gene expression and body weight was observed (Supplemental Table 7). Because *Wnt5a* expression is important in inhibiting the differentiation of mesenchymal stem cells to adipocytes (53), lower *Wnt5a* expression may promote higher body weights. Equally unexpected was the positive correlation between the abundance of unclassified *Clostridiales* and body weight (Supplemental Table 7). Terminal muscle weight (but not epididymal fat weight) also correlated with abundance of this microbial family ($\rho = 0.38$, $P = 0.04$), suggesting that increased body weights were due to lean mass accretion. Further studies are needed to confirm any association of this microbial family with lean body weight.

A positive correlation between terminal muscle weight and apoA-I, HDL cholesterol, and HDL_2 cholesterol was observed (Supplemental Table 8). This supports the findings of Lehti et al. (54), who recently demonstrated that HDL cholesterol is required for normal glucose homeostasis in muscle cells and that apo A-I enhances intramuscular glucose utilization.

Strengths of this study include the use of semipurified diets and an inbred animal model so that the observed microbiota changes were minimally confounded by differences in non-protein components of the diet or differences in the animals' genetic background. Limitations are that the hamster model, although a good model of human cholesterol metabolism, may not fully replicate human metabolism. In addition, the lack of annotation of the hamster genome precluded more extensive analyses of gene expression changes. Although not performed, metabolomic analyses may have permitted a better understanding of the microbiota-driven metabolic changes that led to the observed decreases in lipogenesis in the soy-fed groups.

In conclusion, this study provides strong evidence for the ability of soy protein compared with milk protein to reduce plasma lipids in hamsters at least, in part, by its ability to modulate the gut microbiota. Although the differently processed soy proteins showed some variability in microbial profiles, the microbial profiles in soy-fed hamsters were more closely related to each other and significantly different from those seen in the MPI-fed group.

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