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Original Article

Differential Effects of Sulfur Amino Acid-Restricted and Low-Calorie Diets on Gut Microbiome Profile and Bile Acid Composition in Male C57BL6/J Mice

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Abstract

Diet can affect health and longevity by altering the gut microbiome profile. Sulfur amino acid restriction (SAAR), like caloric restriction, extends lifespan. But, its effect on the gut microbiome profile and functional significance of such effects are understudied. We investigated whether SAAR alters the gut microbiome profile and bile acid composition, an index of microbial metabolism. We also compared these changes with those induced by a 12% low-calorie diet (LCD). Male 21-week-old C57BL6/J mice were fed control (CD; 0.86% methionine), SAAR (0.12% methionine), and LCD diets (0.86% methionine). After 10 weeks on the diet, plasma markers and fecal microbial profiles were determined. SAAR mice had lower body weights and IGF-1, and higher food intake and FGF-21 than CD mice. Compared to SAAR mice, LCD mice had higher body weights, and lower FGF-21 and food intake, but similar IGF-1. β-Diversity indices were different between SAAR and LCD, and LCD and CD, but not between CD and SAAR. In groupwise comparisons of individual taxa, differences were more discernable between SAAR and LCD than between other groups. Abundances of Firmicutes, Clostridiaceae, and Turicibacteraceae were higher, but Verrucomicrobia was lower in SAAR than in LCD. Secondary bile acids and the ratio of secondary to primary bile acids were lower in SAAR than in LCD. SAAR favored bile acid conjugation with glycine at the expense of taurine. Overall, SAAR and LCD diets induced distinct changes in the gut microbiome and bile acid profiles. Additional studies on the role of these changes in improving health and lifespan are warranted.

Keywords: Clostridiales, Firmicutes, Lifespan, Methionine restriction, Sulfur metabolism

Aging is a multifactorial phenomenon. While intrinsic aging (natural physiological decline) is genetically determined, extrinsic aging (ability to cope with external insults) can be modified by various environmental factors, including diet. The gut microbiome, which includes commensal, symbiotic, and pathogenic microorganisms, occupies a unique ecological niche, as it can interact with both the host and its diet. Thus, the microbiome can modify the effect of a diet on the host. However, the community structure (species diversity and relative abundance of the constituent taxa) of the microbiome itself depends on the host's diet. Any changes in this tight-knit triad can have beneficial or detrimental effects on the health of the host. Longstanding interest on the impact of dietary macronutrients on specific microbial taxa and consequences on health has generated significant knowledge (1,2). But, the effect of subtypes of macronutrients such as the types of fats, proteins, and individual amino acids on microbial ecology and their association with the progression of systemic disorders and aging is only beginning to emerge. Sulfur amino acid restriction (SAAR, restriction of dietary methionine in the absence of cysteine, also called methionine restriction) and caloric restriction are the 2 most consistently demonstrated dietary interventions that extend lifespan in laboratory models (3,4). A mechanistic role is suggested for the gut microbiome in caloric restriction-mediated lifespan extension through energy balance and immune homeostasis (5,6). However, the magnitude of such effects is yet to be determined. Whether SAAR alters the gut microbiome, and if so, the contribution of these changes to SAAR-induced bene-

fits is unknown. Although limited, evidence from existing literature on bacterial sulfur metabolism, the interaction between the amino acid metabolisms of the host and the microbiome, and findings from in vivo studies suggest that SAAR modifies the gut microbial profile (7,8).

Bacteria, despite their capacity to synthesize sulfur amino acids from inorganic sulfur, consume methionine and cysteine from the natural environment and alter their growth and metabolism based on the exogenous availability of the 2 sulfur amino acids (9). MetJ, a transcription factor in bacteria, coordinates the expression of methionine transporters (MetD, MetP, MetQ, and MetI) in a facultative fashion (10). However, the biochemical pathways for methionine biosynthesis, sensitivity, and metabolic adaptation to exogenous methionine are variable among different classes of bacteria (11,12). For instance, in the presence of exogenous methionine, methionine biosynthetic enzymes are repressed in certain bacterial species but not in others (13). While most enteric bacteria can use either methionine or cysteine as the sole source of sulfur, some bacteria such as *Escherichia coli* and *Salmonella enterica* can only convert cysteine to methionine, but not vice versa (14,15). *Mycobacterium*, which can use both, prefers exogenous methionine as a source of sulfur (16). In others, despite the lack of effect on growth, exogenous methionine is indispensable for chemotaxis (17,18). This variability in the dependence on sulfur amino acids suggests that SAAR can alter both the microbial profile and their metabolism.

A limited number of studies have investigated the effect of dietary sulfur amino acids on the gut microbiome. Germ-free CD-1 mice required lower levels of dietary methionine compared to mice raised in a conventional animal facility to achieve similar growth rates (19). A study in piglets showed similar findings where parenteral requirements of methionine were 30% lower compared to enteral requirements (20). Thus, the ingested methionine is assimilated, not just by the host but also by its microbiome. Dietary methionine concentration also alters the abundance of different pathogenic bacteria in broiler chicks (7). Other studies have documented symbiosis between the metabolisms of the host and its microbiome in response to the changes in the dietary availability of methionine. *Buchnera*, an endosymbiotic bacterium in aphids, increases methionine biosynthesis as an adaptive response to the lack of methionine in its host's diet (21). While these studies show that gut microbiome alter their metabolism based on the dietary methionine content of their host, other studies suggest that such changes can impact the lifespan of the host. The methionine biosynthetic ability of *E. coli*, which is used as food for *Caenorhabditis elegans*, can be altered by genetic mutations. Feeding *C. elegans* with mutant *E. coli* that cannot synthesize methionine results in a metabolic phenotype associated with lifespan extension akin to SAAR (22). Despite the existence of strong evidence that SAAR could change gut microbiome profile and that these changes could have functional significance, they are not yet characterized.

As an initial step towards this goal, we conducted an observational study to investigate whether SAAR alters the gut microbial profile in male C57BL/6J mice by feeding either a control diet (CD; 0.86% methionine without cysteine) or an SAAR diet (0.12% methionine without cysteine) for 10 weeks. Despite having ad libitum access, the total caloric intake of animals on SAAR is documented to be lower than that on the control diet (23). In order to eliminate the confounding effects of decreased caloric intake, we included another group of mice, which were fed the CD diet, but total caloric intake was matched to that of SAAR diet (low-calorie diet [LCD]). To identify any metabolic implications of altered microbial profile on the host, we also determined plasma bile acid profiles, a marker of microbial metabolism.

Method

Animals and Diet

Animal procedures were conducted following the guidelines of the Institutional Animal Care and Use Committee of the Orentreich Foundation for the Advancement of Science. Male C57BL/6J mice (12 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were received group-housed in shipping containers during the transport. Immediately after receiving, mice were housed in individual cages and quarantined for a week. They were then single-housed in polycarbonate cages in a dedicated room of a conventional animal facility until the end of the study. The housing conditions were 50% ± 10% relative humidity, 20 ± 2°C, and 12-hour dark/12-hour light cycle. Until they reached the age of 21 weeks, mice were fed Laboratory Rodent Diet 5001 (PMI Nutrition International, Brentwood, MO) and offered acidified water ad libitum. At 21 weeks of age, mice were randomized into 3 diet groups: CD (ad libitum intake of CD diet), SAAR (ad libitum intake of SAAR diet), and LCD (CD diet with total caloric intake matched to that of the SAAR group). Fresh food was offered to CD and SAAR mice every week and every day for LCD mice for a total duration of 10 weeks. During the first week, LCD mice were fed ad libitum. From the second week onwards, LCD mice were pair-fed to the food intake of SAAR mice. The weekly food intake of LCD mice was matched to the average food consumption of SAAR mice during the preceding week. The food was rationed into 7 equal portions and 1 portion was offered daily during the day time. Based on paired-feeding to SAAR, the total caloric intake of the LCD mice was 88% of the caloric intake of the CD mice, that is, a caloric restriction of 12%. The compositions of the CD and SAAR diets are given in Supplementary Table 1. The number of mice in each group ranged from 7 to 9. At the end of the study, mice were fasted for 7 hours and bled by the retro-orbital route and sacrificed by CO_2 asphyxiation, followed by cervical dislocation. For plasma collection, blood was immediately centrifuged at 15 000*g* and 4°C for 10 minutes. Plasma was stored at −80°C until analyzed for bile acid profiles.

Plasma Protein Concentrations

Plasma IGF-1 and FGF-21 concentrations were quantified by ELISA following the manufacturers' protocols. For IGF-1, a Mouse/Rat IGF-1 kit (Catalog # MG100, R&D Systems, Minneapolis, MN) was used. Plasma was diluted 500-fold in the supplied Calibrator Diluent solution. Optical densities (ODs) were measured at 450 nm with a reference wavelength of 540 nm on a Spectramax M5 spectrophotometer (Molecular Devices, San Jose, CA). Plasma IGF-1 concentrations were obtained by using a 4-parameter logistic curve built with corrected ODs and known concentrations of analytes.

FGF-21 was measured with a Rat/Mouse FGF-21 Kit (Catalog # EZRMFGF21-26K, MilliporeSigma, St. Louis, MO). Based on our experience with previous studies and to save on the limited volumes of plasma, 10 µL of samples were used in the assay either as undiluted (CD and LCD) or after a 2-fold dilution with assay buffer (SAAR). The ODs were read at 450 nm with a reference wavelength of 590 nm. Similar to IGF-1, FGF-21 concentrations were determined by using a 4-parameter logistic curve.

Microbiome Profiling

Stools were collected 1 week before the sacrifice. Mice were transferred to clean cages without bedding for up to 2 hours and allowed to freely defecate. Contamination during the collection of fecal pellets was prevented by housing mice in individual cages, continuously monitoring the mice, and moving to new cages upon urination. After collecting 4–6 pellets each, mice were returned to their original cages. Stools were immediately frozen in liquid nitrogen and stored at −80°C until DNA extraction. PowerFecal DNA Isolation Kit (Qiagen, Germantown, MD) was used to obtain bacterial DNA and was extracted following the manufacturer's recommendations. DNA concentration was determined by using QuantiFluor's dsDNA System (Promega, Madison, WI), and 1 µg of DNA was used for microbial profiling.

Microbial profiling was performed at Second Genome (South San Francisco, CA). Profiling was based on the identification of unique sequences within highly conserved regions of the 16S V4 rRNA gene. Bacterial DNA was PCR-amplified using fusion primers consisting of complementary sequences for conserved regions and tagged with indexing barcodes. PCR products from all samples were pooled and sequenced in a MiSeq sequencer for 250 cycles with paired-end sequencing primers (San Diego, CA). Raw sequences were quality-filtered with USEARCH, and the resulting unique sequences were clustered at 97% similarity by UPARSE (de novo operational taxonomic unit [OTU] clustering) (24). Sequences were then mapped (at 99% similarity) to a set of representative sequences from the Greengenes reference database of 16SrRNA, and an OTU abundance table was generated. OTUs were assigned by using Mothur's Bayesian classifier.

Bile Acid Profiling

Plasma bile acid profiling was performed by UPLC-MS/MS at Albert Einstein College of Medicine (The Bronx, NY). Detailed methods are available elsewhere (25). Briefly, proteins in 25 µL of plasma were precipitated by adding 100 µL of a methanol:acetonitrile mixture (5:3 v/v) and incubating on ice for 15 minutes. Samples were centrifuged at 10 000*g*, and 10 µL of supernatants were injected into a UPLC-MS/MS system (Xevo TQ, Waters, Pittsburgh, PA). The UPLC was equipped with a C18 column (1.7 µm, 100 mm × 2.1 mm ID; Waters, Milford, MA) and maintained at 40°C. Mobile phase A consisted of 10 mM ammonium acetate at pH 4, while mobile phase B was pure methanol. The elution conditions were as follows: isocratic at 40% B (0–0.5 minutes), followed by linear gradients from 40% to 80% B (0.5–9.0 minutes), 80%–100% B (9.0–12.0 minutes), isocratic at 100% B (12.0–12.5 minutes); and isocratic at 40% B (12.5–15.0 minutes) with a flow rate of 0.3 mL/min. Mass spectrometry was performed by a Xevo TQ-S mass spectrometer fitted with an ESI source in negative ion mode. The mass spectrometer was operated with the source and desolvation temperatures set at 120°C and 350°C, respectively, and the desolvation gas (nitrogen) was set at a flow rate of 650 L/h. The entire LC–MS system was controlled by MassLynx 4.1 software. Proper quality control measures were followed during the analysis. Glycocholate-d4 (125 ng/mL) was added as an internal standard to all samples. Two pooled plasma samples were run as quality controls before, in the middle of, and after the individual samples were run. Bile acids with a coefficient of variation more than 20% within quality control samples were not considered for data analysis. A mixed standard was made by mixing γ-muricholate, glycochenodeoxycholate, glycocholate, glycodeoxycholate, glycohyodeoxycholate, glycolithocholate, glycoursodeoxycholic acid, lithocholate, tauro α, β-muricholate, taurochenodeoxycholate, taurocholic acid, taurodeoxycholate, taurohyodeoxycholate, taurolithocholate, and ursodeoxycholic acid. The mixed standard was further diluted in methanol to obtain different concentrations ranging from 0.01 to 10 000 ng/mL and included in the analysis before and after the samples were run. Raw data obtained were analyzed using QuanLynx 4.1 application manager. Classification of individual bile acids into primary or secondary, and conjugated or unconjugated is presented in Supplementary Table 2.

Statistics

GraphPad Prism 8 was used for data analysis of morphometrics, plasma protein concentrations, bile acid composition, and percent abundance of microbiome OTUs (phylum, family, and species). Simple mean differences among groups were analyzed by 1-way analysis of variance followed by Tukey's multiple comparison test. If the percent abundances of any taxa were less than 0.01%, they were eliminated. Grubb's test was used to detected and omit any outliers from statistical analysis. In all cases, 2-tailed *p*-values less than .05 were considered statistically significant.

Data for differences in microbiome profile between interventions were analyzed in multiple stages using R (version 3.6.1 [2019-07-05]). The "vegan" package in R is extensively used for microbiome analysis (26). In the first stage, simple principal coordinates analysis was used to visualize similarity or nonsimilarity among the microbiome profiles. To quantify the richness of the relative abundance data, α-diversity measure was calculated using "Chao1 Richness," whereas β-diversity was calculated using Bray–Curtis distance to quantify dissimilarity in microbiome composition between groups. In the second stage, 3 simple nonmetric multidimensional scaling plots were created to observe pairwise group difference based on abundance matrices. To compliment this graphical approach, nonparametric analysis of similarity tests were conducted to test differences between groups. *p*-Values were obtained by using 1000 permutations in each test. In the third stage of analysis, we aimed to find the OTUs whose mean abundance significantly differed in each of the 3 pairwise between-group comparisons. Since the number of OTUs were very large, false discovery rates would have been very high, if all OTUs are considered for analysis. To counteract that effect, 3 separate pairwise machine learning-based models were used to find the initial set of OTUs which can differentiate between groups. In each model, the outcome was the group indicator and the predictors were the abundance data for all the OTUs. This strategy enabled us to choose a much smaller set of OTUs, which have a strong predictive power to distinguish between groups. A tree-based machine learning tool called XGBoost was used in that end. XGBoost is a very well-known tool that has been used extensively in multiple fields for binary group comparisons (27). Variable importance was measured using "gain" which quantifies and ranks OTUs based on their predictive contribution (28). In all 3 models, OTUs were chosen if their individual "gain" was more than 0.1%. Finally, in the fourth stage of analysis, the mean abundance of the selected OTUs in stage 3, were compared using robust linear regression in each pairwise group comparisons. The raw *p*-values were corrected for multiple comparison errors by *p*-value adjustment function "fdr." The OTUs that were significantly different in between-group comparisons after adjusting for *p*-values were then visualized with respect to their corresponding class and order level.

Results

Phenotypic Effects of SAAR and LCD Diets

During the first 3 weeks, mice adjusted to each diet differently. CD mice neither gained nor lost body weight during the first 3 weeks, while SAAR and LCD mice lost body weight (Figure 1A). SAAR mice lost significantly more weight than LCD mice. From the third week onwards, CD mice gained weight throughout the study period. LCD mice also continued to gain weight throughout the study period but less than CD mice. SAAR mice neither lost nor gained weight from the third week onwards. The cumulative weight gains from the third week onwards were in the order of SAAR < LCD < CD (Figure 1B, weight gain was −0.006 ± 0.04, 0.45 ± 0.10, and 0.64 ± 0.16 g/wk, respectively) with the SAAR group significantly different from the others ($p < .0001$). On a body weight basis, SAAR mice ate significantly more than CD mice (Figure 1C and D; SAAR/CD: 1.07 g/wk/g body weight; $p < .05$). However, the absolute food consumption in SAAR mice on average was 0.88-fold of that in CD (Supplementary Figure 1, $p < .0001$). Weight gain and food intake observed in our study are similar to previous observations (3,29). Both SAAR mice and LCD mice had lower IGF-1 levels compared to CD (Figure 1E; SAAR/CD: 0.71, LCD/CD: 0.75, p at least ≤ .001). IGF-1 levels in SAAR and LCD were similar. Plasma FGF-21 was higher in SAAR compared to both CD and LCD (Figure 1F; SAAR/CD: 1.69, SAAR/LCD: 2.80, p at least ≤ .05). Even though LCD had lower FGF-21 than CD, the difference was not statistically significant (LCD/CD: 0.60, $p > .05$).

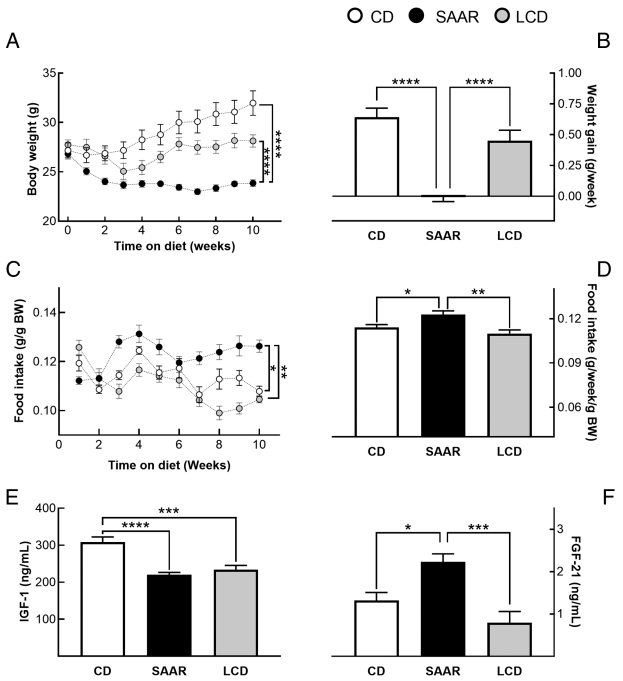

Figure 1. Differential effects of low-calorie diet (LCD) and sulfur amino acid-restricted diet (SAAR) on morphometrics and plasma markers. Male 21-week-old C57BL6/J mice were fed a control diet (CD, 0.86% methionine without cysteine with ad libitum intake); SAAR (0.12% methionine without cysteine with ad libitum intake); and LCD (CD with total caloric intake matched to the SAAR group) for 10 weeks. (**A**) Body weights during the study period, (**B**) cumulative weight gain, (**C**) food intake on a body weight basis, (**D**) cumulative food intake on a body weight basis, (**E**) plasma IGF-1, and (**F**) plasma FGF-21. *Notes*: n = 7–9 per group; error bars represent standard errors. *$p \le .05$, **$p \le .01$, ***$p \le .001$, ****$p \le .0001$.

Microbiome Characteristics

Differences in bacterial composition were found both at whole-community and individual taxonomic levels. No differences were found in the number/richness of the taxa (α-diversity, data not presented). Principle component analysis of Bray–Curtis distance metrics revealed significant differences in β-diversity among the 3 diet groups (Figure 2A; $p = .001$). Groupwise comparisons show that CD and SAAR had similar β-diversity indices (Figure 2B). However, significant differences were found between SAAR and LCD (Figure 2C; $p = .03$), and between LCD and CD (Figure 2D; $p = .005$).

Considering all the diet groups together, the 3 most abundant phyla detected in filtered sequences were Verrucomicrobia (48%), Firmicutes (31%), and Bacteroidetes (19%). The mean abundance of Bacteroidetes was similar in all the 3 diet groups (data not shown). Firmicutes were significantly higher in SAAR than in LCD (SAAR/LCD: 2.99; $p = .002$; Figure 3A). The ratio of abundances of Firmicutes to Bacteroidetes was 2.9-fold higher in the SAAR mice than in LCD mice ($p = .0008$; Figure 3B). Verrucomicrobia in SAAR were less abundant compared to LCD, but similar when compared to CD (SAAR/LCD: 0.53, $p = .02$, Figure 3C). CD and LCD also had similar abundances of Verrucomicrobia. The most abundant families were Verrucomicrobiaceae, Erysipelotrichaceae, Turicibacteraceae, Clostridiaceae, Ruminococcaceae, and Lachnospiraceae, in that order. Significant differences were found in the abundances of 3 families. Clostridiaceae were 4-fold ($p = .005$) and 10.5-fold ($p = .0009$) more abundant in SAAR compared to CD and LCD, respectively (Figure 3D). Turicibacteraceae were 15-fold ($p = .02$) and 25-fold ($p < .0001$) higher in CD and SAAR, respectively compared to LCD (Figure 3E). Verrucomicrobiaceae was 1.8-fold more abundant in LCD compared to SAAR ($p = .01$; Figure 3F).

In terms of species differences among the diet groups, the percent abundance of 94OTU972 (*Clostridium paraputrificum*) was

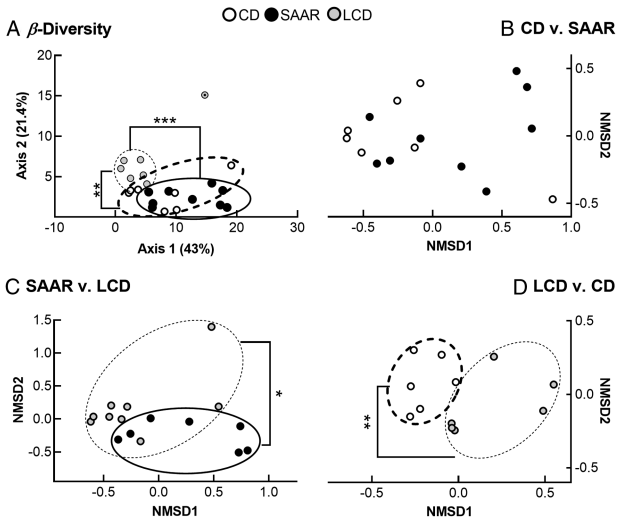

Figure 2. Low-calorie diet (LCD) and sulfur amino acid-restricted diet (SAAR) alter β-diversity of the gut microbiome. (**A**) Differences in the microbiome profiles (β-diversity) of the 3 dietary interventions were calculated using Bray–Curtis distance matrix. (**B–D**) To quantify dissimilarity in microbiome composition between groups, plots and *p*-values were generated by using nonmetric multidimensional scaling and nonparametric analysis of similarity, respectively. β-Diversity indices were not different between (B) control diet (CD) and SAAR, but significantly different between (C) SAAR and LCD, and (D) LCD and CD. *Notes*: n = 7–9 per group. Circle with dot indicates outlier in the LCD group. *$p \le .05$, **$p \le .01$, *** $P \le .001$.

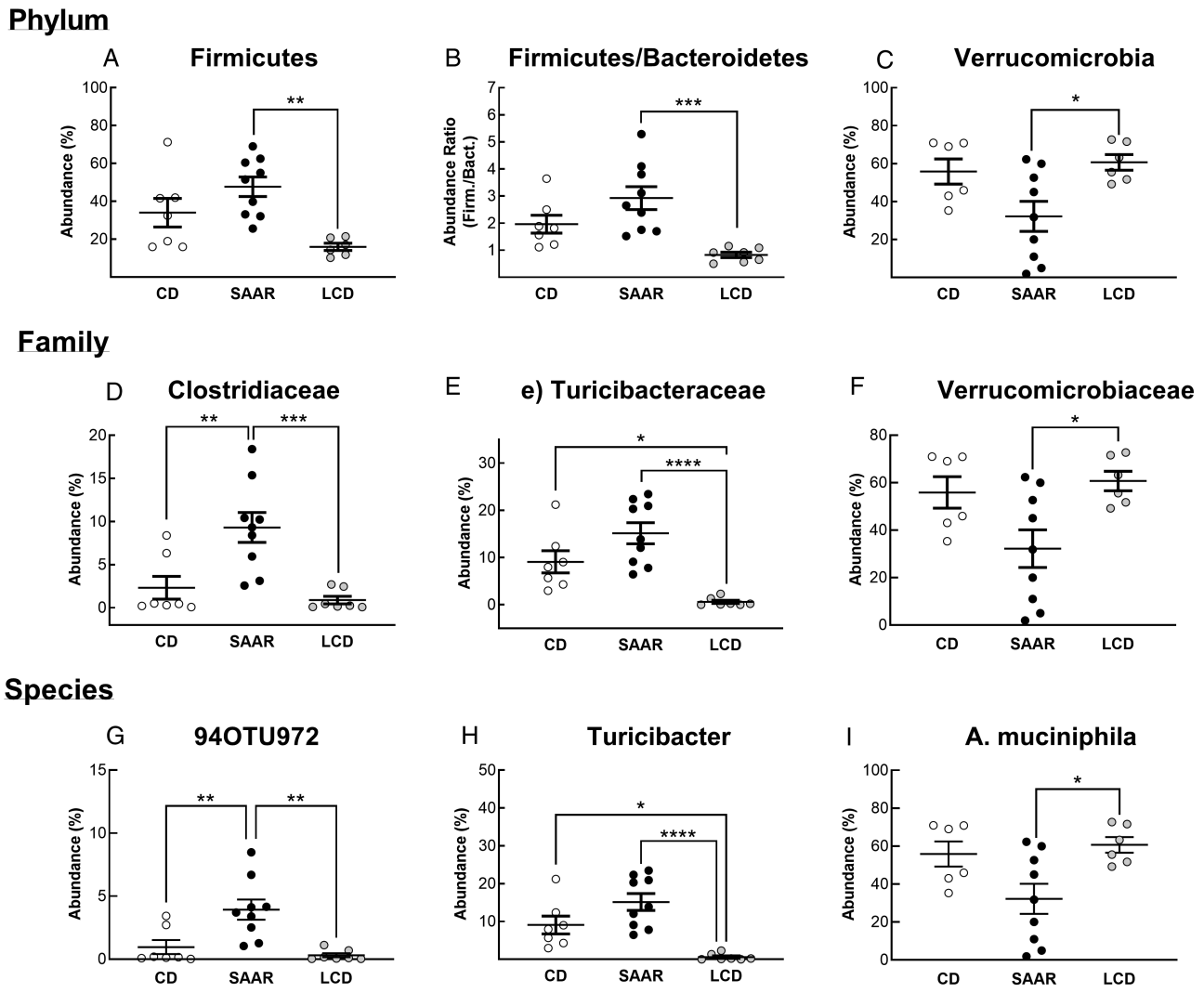

Figure 3. Differential effects of low-calorie diet (LCD) and sulfur amino acid-restricted diet (SAAR) on the mean abundances of selected taxa. (**A–C**) Phylum, (**D–F**) family, and (**G–I**) species. Overall, groupwise comparisons of selected taxa show that SAAR and LCD were the 2 diet groups that were most different. *Notes*: Percentage represents percent of total bacteria; error bars represent standard errors; $n = 7–9$ per group. *$p \le .05$, **$p \le .01$, ***$p \le .001$, ****$p \le .0001$.

significantly higher in SAAR compared to CD and LCD groups (Figure 3G; SAAR/CD: 4-fold, $p = .007$ and SAAR/LCD: 25-fold, $p < .0001$), but there was no difference between CD and LCD. Compared to LCD, Turicibacter was 15-fold more abundant in CD ($p = .02$), and 25-fold more abundant in SAAR ($p < .0001$; Figure 3H). However, SAAR and CD mice had similar abundances of Turicibacter. SAAR mice had significantly fewer *Akkermansia muciniphila* than the LCD mice (Figure 3I; $p = .02$). While the abundance was 0.5-fold lower in SAAR than in CD, this difference was not statistically significant, nor was there a difference between CD and LCD groups. Additional pairwise comparisons of all OTU abundances using robust linear regression revealed significant differences at the OTU levels class and order. The number of OTUs that were significantly different was in the order CD versus SAAR > SAAR versus LCD > LCD versus CD (Supplementary Figure 2). Analyses for family and genus were not conducted since the number of categories were too large to conduct any meaningful comparison for those modest number of significantly different OTUs, whereas the analysis based on phylum revealed overwhelming presence of Firmicutes in all pairwise comparisons.

Bile Acid Profile

Total bile acid concentrations were approximately 2-fold higher in SAAR mice than in CD mice, but not statistically different (Figure 4A). No differences were observed in primary bile acid concentrations (data not presented). Secondary bile acids were 2-fold lower in SAAR mice compared to LCD mice (Figure 4B; $p = .02$). As a result, the ratio of secondary to primary bile acids exhibited a 3-fold decrease in SAAR mice compared to LCD mice (Figure 4C; $p = .04$). CD mice and SAAR mice had similar concentrations of secondary bile acids and the ratio of secondary to primary bile acid concentrations. No differences were observed in the levels of total conjugated bile acids (Figure 4D); however, taurine-conjugated bile acids in SAAR were 0.92-fold of both CD and LCD (Figure 4E; $p \le .01$). In contrast, glycine-conjugated bile acids in SAAR were 12.5-fold ($p = .009$) and 17.7-fold ($p = .01$) higher than in CD and LCD, respectively (Figure 4F).

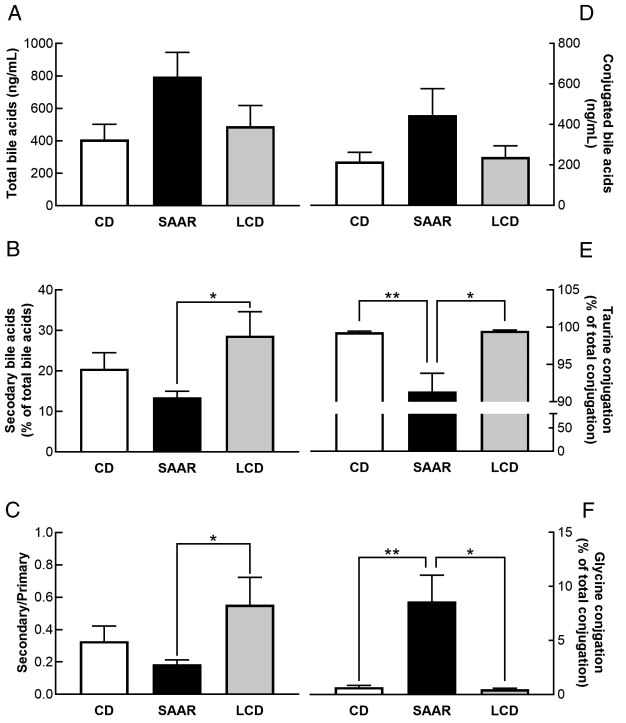

Figure 4. Sulfur amino acid-restricted diet (SAAR) alters plasma concentration (**A–C**) and conjugation (**D–F**) of bile acids. (A) Plasma total bile acids, (B) plasma secondary bile acids, (**C**) ratio of secondary/primary bile acids, (D) total conjugated bile acids, (E) taurine-conjugated bile acids, and (**F**) glycine-conjugated bile acids. *Notes*: $n = 7–9$ per group; error bars indicate standard errors. $*p \leq .05$. $**p \leq .01$.

Discussion

In this study, we examined whether the SAAR diet changes the gut microbial profile and if such changes have any metabolic implications. In addition, we also compared SAAR-induced changes to the changes induced by a mild caloric-restricted diet using LCD. No differences were found in the α-diversity of the fecal microbial profiles. But, β-diversity indices were significantly different. Groupwise comparisons show that β-diversity was different between LCD and SAAR, and LCD and CD, but not between CD and SAAR. At individual taxonomic levels, maximal number of differences were found between SAAR and LCD (phyla Firmicutes and Verrucomicrobia, and families, Clostridiaceae, Turicibacteraceae, and Verrucomicrobiaceae), followed by CD and SAAR (family Clostridiaceae and the species 94OTU972), and CD and LCD (family Turicibacteraceae and species *Turicibacter*). Plasma secondary bile acids and the ratio of secondary to primary bile acids in SAAR were significantly lower compared to LCD. SAAR increased the conjugation of bile acids with glycine at the expense of taurine. Overall, our findings suggest that SAAR diet alters the abundances of certain bacterial taxa and that the changes in the fecal microbial profile induced by SAAR are distinct from the changes induced by LCD.

To our knowledge, very few studies investigated the effects of SAAR on gut microbial profile (30,31). Findings from these studies are significantly different from ours. But, these studies also differ from ours in critical aspects of experimental design, including dietary formulation, age-at-onset, and the gut compartment in which the microbiome profile was determined. For instance, Wallis et al. found a decrease in the abundance of Firmicutes in contrast to the higher trend we observed (30). However, the SAAR diet formulated by Wallis et al. consisted of cysteine at 0.35% while our diet was devoid of cysteine. We formulated our diet in the light of previous findings that the presence of cysteine abrogates certain phenotypes typical of SAAR (32). In another study, Yang et al., formulated the SAAR diet without cysteine, and found a significant increase and decrease in the abundances of Firmicutes and Verrucomicrobia, respectively (31). These changes are similar in trend but stronger than the differences in our study, perhaps due to the difference in the gut compartment in which the microbiome was profiled. To allow for proper comparison with future SAAR interventions in humans and considering the easy access to human stools, we determined microbiome profile in the stools of mice while previous studies used cecal contents (30,31,33). It is well documented that the microbiome profile and ecology depends on the specific compartment of the gastrointestinal tract (34). Additional studies are required to understand the effect of gut compartment on SAAR-induced changes in microbiome profile.

SAAR-induced changes in Firmicutes and Verrucomicrobia reflect bacterial adaptation to the nutrient availability in the gut. Sulfur amino acid metabolism in bacteria is diversified (11). The 2 most common pathways for methionine biosynthesis from homoserine in bacteria are transsulfuration and direct sulfhydrylation pathways. Depending on their genetic makeup, bacteria can utilize either cysteine or inorganic sulfate as the source of sulfur moiety in de novo methionine biosynthesis. The changes in bacterial abundances we observed reflect the biochemical ability of taxa to adapt to the unique gut environment that the SAAR diet presents, that is, low concentrations of methionine and complete absence of cysteine. It was previously reported that except for a few species, bacteria in the phylum Firmicutes synthesize methionine through direct sulfhydrylation and do not require cysteine (12). Thus, the increased abundance of the phylum Firmicutes and its family, Turicibacteraceae, could be due to their metabolic advantage to survive in the absence of cysteine in the gut. The phylum Verrucomicrobia was identified relatively recently and is composed of only a few bacterial species (35). The low abundance of *A. muciniphila*, which represents family Verrucomicrobiaceae, in SAAR is of considerable interest as it feeds extensively on intestinal mucus (36). Low dietary concentrations of sulfur amino acids in pigs are known to decrease the number of goblet cells, which secret mucin, potentially contributing to the decreased abundance of *A. muciniphila* (37).

Unlike the free amino acid diets in rodents, palatability issues in humans necessitate the use of whole foods made of proteins. Thus, the bioavailability and luminal concentrations of amino acids across the gut should be considered in extrapolating findings in rodents to human studies. In addition to small intestine, the large intestine, where the bulk of gut microbiome reside, is a significant site of amino acid absorption. ^{1}H-NMR studies indicate that the rate of transfer of free amino acids across cecum and colon are comparable to the transfer rates across small intestine and that the expression of amino acid transporters is at least as abundant as or higher than that in small intestine (38). Studies in mice show that elemental diets with free amino acids not only alter the microbiome profile adherent to colonic mucosa and in feces, but also reduce bowel inflammation in a colitis model (39,40). Similar alterations of gut microbiome profile and benefits in Crohn's disease were found in humans fed elemental diets with free amino acids (41). These studies clearly demonstrate that the elemental diets including the SAAR diet used in the current study can alter the fecal microbiome profile. However, the effect of an SAAR diet made with intact protein, as often used in human studies, could be different due to the differences in the proportion of amino acids reaching the large intestine.

The total pool size and the composition of individual bile acids are associated with gut microbiome profile and metabolism (42). Plasma bile acid concentrations in SAAR, even though statistically not significant, were approximately 2-fold greater than in CD. This is of considerable importance, as bile acid synthesis is a major catabolic route for cholesterol, and increased synthesis might contribute to lower plasma cholesterol observed in SAAR (43). Supporting our hypothesis, a recent study demonstrated that SAAR increases hepatic mRNA expression of *Cyp7A1*, the rate-limiting enzyme for bile acid synthesis (33). Higher abundance of Firmicutes could be secondary to higher concentrations of bile acids as previous studies demonstrate that supplementing cholic acid in rats increases the abundance of Firmicutes from 54% to 98% (44). In addition to serving as detergents in fat digestion, bile acids can affect other aspects of health. In particular, secondary bile acids and H_2S generated by gut microbiome from taurine-conjugated bile acids are implicated in colon cancer (45,46). Previous studies document that SAAR inhibits colon cancer (47). Considering the lower levels of both secondary bile acids and taurine-conjugated bile acids, the interaction between bile acids and the gut microbiome in prevention of colon cancer by SAAR is an attractive area for future investigations.

Specific microbiome profiles and taxa are associated with extended lifespan in animal models and humans. The ratio of abundances of Firmicutes to Bacteroidetes is known to change throughout development and aging (48). The ratios were 0.4, 10.9, and 0.6 in infants, adults, and the elderly, respectively (48). Thus, the higher ratio, a characteristic feature of gut microbiome in young individuals, observed in SAAR might be contributing to the extension of lifespan. Interestingly, centenarians have a unique microbiome profile compared to short-lived individuals. Species belonging to Enterobacteriaceae, Bifidobacterium, and Bacteroidetes decreased while *Clostridia* sensu was increased (49). It is noteworthy that SAAR also increased the abundance of Clostridiaceae.

Our study, which provides a proof-of-concept for the role of gut microbiome in SAAR-induced benefits, has certain strengths and limitations. Our findings are characteristic of the lifespan–extending SAAR diet, methionine restriction in the absence of cysteine. Absolute food consumption in rodents on the SAAR diet is lower than those fed control diet ad libitum. Thus, findings could be confounded by not just the quality of diet but the caloric intake as well. By adding LCD, which was pair-fed to SAAR, we demonstrate the SAAR-induced effects are not confounded by caloric intake. Pair-feeding of LCD was achieved by offering the CD diet at one particular time of the day. Considering the lack of food during night, it is highly likely that the LCD mice ate all the food offered within a short time. Therefore, there could be some effects associated with time-restricted feeding that cannot be discerned in the current study. Overall, our findings together with recent reports encourage mechanistic investigations in animal models and translational studies to find if SAAR induces similar changes in humans (30,31).

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None declared.

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Author Contributions

S.N.N.: study conception and design, data analysis, and manuscript writing; J.S. and D.A.L.M.: animal husbandry and data acquisition; and V.M.: microbiome profile analysis.

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