Effect of a long-term high-protein diet on survival, obesity development, and gut microbiota in mice

Pia Kiilerich,1* Lene Secher Myrmel,1,2* Even Fjære,1,2 Qin Hao,1 Floor Hugenholtz,2 Si Brask Sonne,1 Muriel Derrien,3 Lone Møller Pedersen,1 Rasmus Koefoed Petersen,1 Alicja Mortensen,4 Tine Rask Licht,4 Maria Unni Rømer,5,6 Ulla Birgitte Vogel,1 Linn Jeannette Waagbo,2 Natasa Giallourou,3 Qiang Feng,8 Liang Xiao,6 Chuan Liu,6 Bjorn Liaset,7 Michiel Kleerebezem,3,9 Jun Wang,1,8,10,11,12 Lise Madsen,1,2,8 and Karsten Kristiansen1,8

1Laboratory of Genomics and Molecular Biomedicine, Department of Biology, University of Copenhagen, Copenhagen, Denmark; 2National Institute of Nutrition and Seafood Research, Bergen, Norway; 3Top Institute Food and Nutrition, Wageningen, The Netherlands; 4National Food Institute, Technical University of Denmark, Søborg, Denmark; 5Department of Veterinary Disease Biology, University of Copenhagen, Copenhagen, Denmark; 6Department of Clinical Physiology, Nuclear Medicine and PET, Rigshospitalet, University of Copenhagen, Denmark; 7National Research Centre for the Working Environment, Copenhagen, Denmark; 8BGI-Shenzhen, Shenzhen, China; 9Host Microbe Interactomics Group, Wageningen University, Wageningen, The Netherlands; 10Princess Al Jawhara Al Bahran Center of Excellence in the Research of Hereditary Disorders, King Abdulaziz University, Jeddah, Saudi Arabia; 11Macau University of Science and Technology, Taipa, Macau, China; 12Department of Medicine and State Key Laboratory of Pharmaceutical Biotechnology, University of Hong Kong, Hong Kong, China

Submitted 5 August 2015; accepted in final form 28 March 2016

INTAKE OF HIGH-FAT DIETS has been associated with the development of obesity and several metabolic dysfunctions, including insulin resistance, hepatic steatosis, hyperlipidemia, and low-grade systemic inflammation (42). Diets with more than 30% energy originating from fat promote obesity in mice, and high-fat diet-induced obesity is a frequently used rodent model for studies on obesity and related metabolic disorders (15). However, these models cannot be used to distinguish whether the observed metabolic dysfunctions result from the obese state or from the high-fat feeding. Rodent studies have shown that obesity is prevented if the increase in dietary fat is accompanied by a high protein-to-sucrose ratio (12, 14, 30, 32, 33, 36). This may reflect higher satiety and diet-induced thermogenesis when the protein content in the diet is increased (31). Conversely, reducing the fat content from 40 to 30 energy% is sufficient to counteract insulin resistance in rodents (16). Together with the finding that glucose intolerance and insulin resistance are detectable within the first week of high-fat feeding, these findings suggest that development of insulin resistance and glucose intolerance may be directly related to the dietary fat content and not to obesity (50).

Even though a high intake of fat may lead to metabolic disturbances prior to the onset of obesity, it is generally acknowledged that obesity reduces longevity (2) and is associated with increased all-cause mortality in humans (11). It is also well documented that energy restriction increases, whereas high-fat feeding reduces lifespan in rodents, but in most experiments it has been difficult to separate the beneficial effects of caloric restriction per se from that of leanness (2). However, the finding that longevity is increased in feed-restricted ob/ob mice despite high levels of obesity suggests that longevity is related to feed intake rather than the state of obesity (17). On the other hand, the increased longevity in fat-specific insulin receptor knockout mice that are protected against diet- and age-induced obesity, despite normal feed intake, suggests that the reduced fat mass, and thereby the possibly reduced burden of obesity-related disorders, may be of importance (2). Moreover, increasing the protein/sucrose ratio increased the longevity in high-fat diet-fed mice, and conversely, decreased longevity was observed in response to a diet with a low protein/sucrose ratio, which was also linked to a high rate of weight gain early in life (22). Still, median

* P. Kiilerich and L. S. Myrmel share first authorship.
Address for reprint requests and other correspondence: K. Kristiansen, Laboratory of Genomics and Molecular Biomedicine, Dept. of Biology, Univ. of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark (e-mail: kk@bio.ku.dk).
lifespan in high-fat/high-protein-fed mice was reduced compared with low-fat-fed mice, and these findings underscore the importance of macronutrient composition. Interestingly, a recent article using a complex geometric framework for nutrition concluded that the ratio of macronutrients, and not caloric intake, was determining various metabolic parameters as well as longevity (43).

Reduced biodiversity and compromised stability of the intestinal microbiota have been reported in elderly humans (46), and modulation of the gut microbiota has been suggested as a modality for longevity extension (34). Dietary composition (8, 19, 47, 49, 51) and the state of obesity (26) are also correlated to the microbial composition in the gut, and recent studies indicate that obesity correlates with decreased microbial gene richness (24). Accumulating evidence indicates that the gut microbiota contributes to the development of diet-induced obesity (1, 8, 40, 47, 48) and additionally influences the development of metabolic dysfunctions associated with obesity (5, 24, 37).

The linkage among diet, obesity, and gut microbiota is, however, not elucidated. On the one hand, the findings that conventionalization of germ-free mice with microbiota from both diet-induced (47) and genetically obese mice fed regular chow (1) resulted in increased weight gain compared with mice transplanted with a gut microbiota from lean controls suggests that obesity drives transferable changes in the gut microbiota, inducing weight gain and adiposity. By contrast, high-fat feeding studies using obesity-resistant RELMβ knockout (KO) mice suggested that dietary factors are more relevant than obesity for microbiome composition (19).

In this study, we investigated the long-term effect of high-fat diets with low and high protein/sucrose ratios on survival, gut microbiota, and the development of obesity and related metabolic disorders in mice. We also hypothesized that this approach would enable us to distinguish whether the observed effects on the gut microbiota reflected the obese state or the high-fat feeding.

**MATERIALS AND METHODS**

*Mice and Feeding*

One hundred fifty female C57BL/6JBomTac mice (3 wk of age, Taconic Europe, Ejby, Denmark) were divided into three experimental groups with 50 mice in each, with an overall equal body weight mean for all the groups. Within each group, mice were randomly separated into 10 cages with five animals in each. Mice were kept at 55 ± 5% humidity, 22 ± 1°C, on a 12:12-h light-dark cycle.

Mice were fed ad libitum a low-fat reference (REF) diet or a corn oil-based high-fat diet (25%) enriched with either protein or sucrose (43%), all obtained from Sniff Spezialdiäten (Soest, Germany) (Table 1) throughout life. Animals were weighed, and feed and water intake were recorded once a week throughout the experiment. All mice that died or the mice the were euthanized because they became moribund or showed markedly disturbed general condition during the study were necropsied. At termination, all the remaining mice were euthanized and necropsied. For each feeding group the experiment was terminated when 50% of the animals had died.

Ten mice from each group were euthanized by cardiac puncture after anesthesia 6 or 18 mo after the start of the experiment. Prior to euthanasia, animals were weighed. Blood was collected and separated into plasma and RBC before freezing at −80°C. Liver, interscapular brown adipose tissue (iBAT), inguinal white adipose tissue (iWAT), and gonadal white adipose tissue (gWAT) were weighed and stored at −80°C until further analyses.

### Table 1. Diet composition

<table>
<thead>
<tr>
<th></th>
<th>Low-Fat Diet (REF)</th>
<th>High-sucrose (HFS)</th>
<th>High-protein (HFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/kg)</td>
<td>200</td>
<td>200</td>
<td>540</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>540</td>
</tr>
<tr>
<td>t-Cystine</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Carbohydrate (g/kg)</td>
<td>619.5</td>
<td>439.5</td>
<td>99.5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>529.5</td>
<td>9.5</td>
<td>9.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>90</td>
<td>430</td>
<td>90</td>
</tr>
<tr>
<td>Fat (g/kg)</td>
<td>70</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Corn oil</td>
<td>180</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>Energy (kcal/kg)</td>
<td>3,908</td>
<td>4,808</td>
<td>4,808</td>
</tr>
<tr>
<td>Protein (E%)</td>
<td>20</td>
<td>16.6</td>
<td>44.9</td>
</tr>
<tr>
<td>Carbohydrate (E%)</td>
<td>63.4</td>
<td>36.6</td>
<td>8.3</td>
</tr>
<tr>
<td>Fat (E%)</td>
<td>16.1</td>
<td>46.8</td>
<td>46.8</td>
</tr>
<tr>
<td>P-to-C ratio</td>
<td>0.32</td>
<td>0.46</td>
<td>5.43</td>
</tr>
</tbody>
</table>

Equal for all diets: cellulose (50 g/kg), choline bitartrate (2.5 g/kg), mineral mix (45 g/kg), vitamin mix (10 g/kg), tert-butylhydroquinone (0.014 g/kg).

Fresh feces was collected from the cages every 2 days at 3, 6, and 16 mo after the start of the experiment.

The experiment was approved by the Animal Experiment Inspectorate in Denmark and was conducted in compliance with the European Convention of Helsinki Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes (Council of Europe, no. 123, Strasbourg, France, 1985).

**Oral Glucose Tolerance Test**

Glucose (1.5 mg/kg body wt) was administered orally in overnight-fasted mice. Blood was collected from the tail vein of conscious animals at 15, 30, 60, and 120 min after glucose administration, and blood glucose was measured using a glucometer (Ascensia Contour, Bayer, Norway) at baseline and during the test at the indicated time points.

**Analyses**

*RNA extraction and RT-qPCR. RNA was extracted from liver, iWAT, and gWAT using TRIzol Reagent (Invitrogen, Carlsbad, CA), cDNA was synthesized in duplicates using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) containing Multiscribe Reverse Transcriptase. Gene expression was determined in 384-well reaction plates by RT-qPCR using the LightCycler 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland). Expression of target genes was normalized to TATA binding protein (Tbp) mRNA. Primer sequences are available upon request.*

*Tissue lipid extraction and lipid class analysis. Total lipid was extracted from liver samples with chloroform–methanol, 2:1 (vol/vol) and quantified on a Camaq HPTLC system and separated on HPTCL silica gel as previously described (30).*

*Plasma lipids. Plasma triacylglycerol and cholesterol were determined using conventional enzymatic kits (DIABAL, Austria) and a MaxMat PL II (MAXMAT S.A., Montpellier, France).*

*Digital gene expression profiling and data analysis. Tag library preparation from liver, iWAT, and gWAT RNA was performed using an Illumina NIIII Gene expression sample preparation kit and sequenced using an Illumina Genome Analyzer II system (BGI-Shenzhen, Shenzhen, China) according to the manufacturer’s recommendations. Image analysis, base calling, and extraction of tags were performed using the Illumina pipeline. The tag entites of digital gene expression (DGE) profiling libraries were mapped to mRNA reference using bwa (27). The matched tags were filtered by the match position to NIIII recognition CATG sites by custom perl scripts. Bioconductor package edgeR (41) was used to perform statistical analyses for
finding genes that differed significantly in expression between diets and time points in different tissues (diets, \( P \leq 0.05 \); time, FDR \( \leq 0.05 \)). GO annotation for Biological processes was performed using the bioconductor package GOstats (10).

**MITChip analysis.** Bacterial DNA was extracted from fecal samples from three to seven cages per diet group as previously described (18). The microbial communities in fecal samples were analysed using the Mouse Intestinal Tract Chip (MITChip). This phylogenetic microarray was designed using criteria of the Human Intestinal Tract Chip (HITChip) developed by Rajilic-Stojanovic et al. (38). The MITChip consists of 3,580 different oligonucleotides specific for the mouse intestinal microbiota (13). The array targets the V1 and V6 regions of 16S rRNA genes of bacteria. The 16S rRNA genes were amplified from 20 ng of intestinal metagenomic DNA with the primers T7prom-Bact-27-F and Uni-1492-R (Table 2). These PCR products were transcribed, labeled with Cy3 and Cy5 dyes, and fragmentated as described elsewhere (38). Finally, the samples were hybridized on the arrays at 62.5°C for 16 h in a rotation oven (Agilent Technologies, Amstelveen, The Netherlands). After washing and scanning of the slides, data were extracted with the Agilent Feature Extraction software v. 9.1. The data were normalized and analyzed using a set of R-based scripts in combination with a customized relational database, which operates under the MySQL database management system.

The RPA signal intensities were taken to analyze microbiota profiles at different levels of taxonomic resolution as indicated in the text (23). Phylum-level data were used to calculate the Bacteroidetes and Firmicutes levels, and differences were tested with Student’s t-test. To determine correlation of genus-like level microbial groups detected on the MITChip with a specific diet, redundancy analysis (RDA) as implemented in Canoco for Windows 5 was used (4). RDA is a linear method of canonical ordination, which linearly combines explanatory variables on the ordination axis. The Monte Carlo Permutation test was used to assess the significance of the variation in the RDAs. Probe-level data were used to calculate the Shannon diversity with an in-house R-script.

**Statistics**

All data are presented as means ± SE. All statistical analyses were carried out in GraphPad Prism, except Digital Gene Expression Profiling and MITChip analysis (see their respective paragraphs for details). Comparisons between groups were performed on all data by one-way ANOVA followed by a Bonferroni adjusted Fisher’s least significant difference (LSD) test, taking into account the total number of pairwise comparisons. For repeated measures of the body weight development curve, a two-way repeated-measures ANOVA was applied to detect overall effects of diet on body weight and time, and post hoc Bonferroni adjusted pairwise comparisons were used to detect weekly differences between diets. Kaplan-Meier survival curves were analyzed using a log-rank test to determine significant difference in survival. In all cases a significance level of \( \alpha = 0.05 \) was used.

**RESULTS**

A *High Dietary Protein/Sucrose Ratio Attenuates High-Fat Diet-Induced Weight Gain and Mortality*

The mice were fed the experimental diets (Table 1) ad libitum from the age of 3 wk and onward until 50% of the mice in each experimental group had died; at that point all the remaining mice were euthanized. A high protein/sucrose ratio strongly attenuated high-fat diet-induced weight gain (Fig. 1A), but high-fat/high-protein (HFP)-fed mice still gained more weight than mice fed the low-fat reference (REF) diet. Mice receiving the high-fat diet supplemented with sucrose (HFS) were significantly heavier than the REF group already at week 10, whereas the difference between the REF- and HFP-fed mice first became significant from week 53. The body weight development revealed distinct differences in growth rates during the feeding trial, and growth rates for HFS-fed mice were higher than for HFP-fed mice during weeks 0–8 and weeks 10–65. As expected, growth rates declined significantly with time for all diets (\( P < 0.0001 \)).

Weekly and cumulative energy intakes were significantly higher in both HF groups compared with the REF group (\( P < 0.0001 \) for all comparisons; data not shown). The HF-fed mice had a comparable energy intake during the feeding trial, with the exception of weeks 4–10 and weeks 70–76, when the HFS-fed mice ate more. This resulted in a significantly higher weekly and cumulative energy intake in the HFS group compared with the HFP group (\( P < 0.0039 \) and \( P < 0.0001 \), respectively). Feed efficiencies were comparable in HFS- and HFP-fed mice during the first 8 wk of feeding (Fig. 1B). However, during weeks 10–65, the feed efficiency in HFS-fed mice, but not HFP-fed mice, was significantly higher than in the REF group. In weeks 70–95, HFS- and HFP-fed mice lost body weight. For all groups, a reduction in feed efficiency was also evident during that time.

The white adipose tissue depots, inguinal white adipose tissue (iWAT) and gonadal white adipose tissue (gWAT), reflected body weight, and mice fed the HFP diet were protected against an increase in adipose tissue mass compared with the REF-fed mice (Fig. 1C). As expected, the WAT increased with time in all groups.

Comparison of the survival curves between the different dietary groups until 50% survival revealed a significant reduction in survival for mice fed the HFS diet compared with the REF group (Fig. 1D). In agreement with Keipert et al. (22), a high protein/sucrose ratio attenuated high-fat diet-induced weight gain; furthermore, the survival of mice fed HFP was not statistically different from that of the REF-fed mice. All deceased animals were subjected to necropsy, but there was no systematic cause of death. Overall, the relationship between diets, adiposity, and survival indicated that obesity, and not the high-fat feeding, was linked to a reduction in survival as determined when 50% of the mice had died. Thus, a high-fat diet led to a significant reduction in survival only when combined with a high sucrose intake that also increased body weight and fat mass. On the other hand, a high-fat diet combined with high-protein intake prevented a significant reduction in survival, and in addition reduced body weight development compared with the HFS diet, and prevented a
significant increase in fat mass compared with the REF-fed mice.

A High Protein/Sucrose Ratio Diminishes High-Fat Diet-Induced Accumulation of Lipids in Liver, but Not a Transient Reduction in Glucose Tolerance

Obesity and high-fat feeding are associated with hepatic steatosis. Mice fed a diet with high protein/sucrose ratio were protected against high-fat diet-induced accumulation of triacylglycerol in the liver after 3 and 6 mo of feeding (Fig. 2A). The HFP-fed mice were also protected from a high-fat diet-induced increase in liver weight compared with the REF-fed mice, contrasting the increased liver weight and triacylglycerol accumulation observed for the HFS-fed mice (Fig. 2A).

In agreement with previous studies in male mice (14, 30, 32), intake of a diet with high protein/sucrose ratio did not protect the mice against high-fat diet-induced impairment of glucose tolerance at 3 mo (Fig. 2B). Both groups of HF-fed mice displayed impaired glucose clearance compared with mice receiving the REF diet, with the HFP-fed mice exhibiting the largest area under the curve during the glucose tolerance test. However, at 18 mo we observed no difference in glucose clearance between the groups (Fig. 2C).
Modulation of Global Gene Expression in Liver and Adipose Tissue during Time Is Dependent on the Protein/Sucrose Ratio

To achieve an overview of global changes in gene expression over time in relation to feed intake and composition, we performed DGE analyses on liver and two metabolically different adipose tissue depots, iWAT and gWAT. For iWAT and gWAT, PCA plots revealed a clear separation between the samples taken at different time points (6 vs. 18 mo), whereas the separation according to the protein/sucrose ratio was less pronounced (Fig. 3A). For liver, only samples from HFP-fed mice separated clearly after 18 mo of feeding. In keeping with the clear clustering in the PCA of gene expression in liver from HFP-fed mice at 18 mo, 361 genes in the HFP-fed mice, but...
only 15 genes in the HFS-fed mice, were differentially regulated between 6 and 18 mo, demonstrating the impact of the HFP diet over time in liver. In the fat depots, the HFS diet caused a higher number of genes to be differentially regulated between 6 and 18 mo in iWAT (663/H11001 150) and gWAT (1,014/H11001 199) compared with HFP feeding (iWAT 154/H11001 150 and gWAT 269/H11001 199), indicating significant diet-dependent differences in expression of genes over time especially in adipose tissues (Fig. 3B).

A High Dietary Protein/Sucrose Ratio Modulates Long-Term Effects on Expression of Genes Involved in Fatty Acid Metabolism, Amino Acid Degradation, and Gluconeogenesis

To investigate whether hepatic lipid accumulation was associated with changes in expression of genes involved in fatty acid synthesis and oxidation, we performed RT-qPCR analyses on RNA isolated from liver. In line with previous short-term studies (30, 32), hepatic expression of the lipogenic genes Acaca, Fasn, and Scd1 was reduced in the HFP-fed mice after 3 mo of feeding (Fig. 4A). However, after 6 mo of HFP feeding, only expression of Scd1 was reduced, whereas expression of both Scd1 and Fasn was reduced after 18 mo of HFP feeding. This demonstrates that diet-induced changes in gene expression persisted for only some of the measured lipogenic genes during long-term feeding, suggesting that a general lower capacity for de novo hepatic fatty acid synthesis in HFP-fed mice was maintained only during the first 3 mo of feeding. Still, the HFP-fed animals remained less obese than the HFS-fed animals throughout the study. By 3 mo of feeding, the levels of mRNAs encoding enzymes involved in fatty acid oxidation (Acot1, Cpt1a, and Acadm) and ketogenesis (Hmgcs2) were similar in low-fat- and high-fat-fed mice (Fig. 4B). By 6 mo, expression of the genes involved in fatty acid oxidation, Acot1 and Cpt1a, was upregulated in the high-fat-fed mice compared with low-fat-fed mice, although this was not seen after 18 mo. Thus, the protein/sucrose ratio strongly affected expression of genes involved in lipid metabolism during the first phases of feeding, but the difference between the HFS- and HFP-fed mice diminished over time.

A sustained increase in mRNA levels of genes involved in amino acid degradation (Got1, Cps1, Agxt, and Gpt; Fig. 4C) and gluconeogenesis (Pck1; Fig. 4D) in liver was observed throughout the experiment in HFP-fed mice, indicative of increased energy-demanding amino acid catabolism in HFP-fed animals compared with HFS-fed animals. This is in keeping with the lower body weight and fat pad mass in the HFP-fed animals.

High-fat feeding and adipose tissue expansion are associated with low-grade inflammation in adipose tissue. HFS feeding led to increased expression of mRNA encoding inflammatory markers, Ccl2 [chemokine (C-C motif) ligand 2] and Serpine1 [serine (or cysteine) peptidase inhibitor, clade E, member 1] (Fig. 4E), but not in macrophage infiltration markers Cd68 and Emr1 (EGF-like module containing mucin-like, hormone receptor-like sequence 1) at 3 and 6 mo (Fig. 4F). This increase was attenuated in mice fed the diet with the high protein/sucrose ratio. However, after 18 mo of feeding, expression levels were similar in all groups, except that expression of Cd68 and Emr1 mRNA was elevated in HFP-fed mice compared with HFS- and REF-fed mice.
Microbiota Composition

Whereas changes in gene expression can reflect modulation of metabolic processes, several recent studies have suggested that the gut microbiota plays an important role in diet-induced obesity (1, 8, 24, 40, 47, 48) and associated metabolic disorders (24, 37). To investigate how host age, dietary fat, protein/sucrose ratio, and obesity impact on the bacterial composition in the gut, bacterial DNA from feces (n = 3–6 per diet group and time point) was analyzed using the Mouse Intestinal Tract Chip (MITChip) (13, 45).

First, as expected, the high-fat diets resulted in a distinct microbiome clustering compared with the REF diet (Fig. 5). Comparison of the microbiomes in the three dietary groups using redundancy analysis (RDA) (Fig. 5) confirmed that particularly the high fat content of the HFS and HFP diets affected the gut microbiome composition. Separation of the high-fat diets from the REF diet was predominantly driven by phylotypes within the Lactobacillus genus (L. delbrueckii, L. plantarum), which were common for the high-fat diets at all three time points (Fig. 5). The protein/sucrose ratio also affected the gut microbiome composition (Fig. 6), where a high abundance of E. cylindroides and the genus-like group Eggertella et rel. characterized mice fed the HFS diet, whereas phylotypes within the Clostridiaceae family (Anaerovorax, Bryantella, C. herbivorans, C. sphenoides, C. leptum, and C. symbiosum) characterized mice fed the HFP.

Second, the aging of the mice also resulted in distinct differences in the microbial communities. The Firmicutes/Bacteroidetes (F/B) ratio decreased with age in the mice fed REF and HFP diets, whereas no significant change was observed in mice fed the HFS diet (not shown). RDA plots showed distinct separation of the microbiomes from 3 over 6 to 16 mo of feeding for all three diets (Fig. 7, A–C). Interestingly, although there was no common bacterial phylotypes driving the age-related separation of the gut microbiomes across the diets at 3 mo, several phylotypes (Sphingomonas, Desulfovibrio, Olsenella, Akkermansia muciniphila) were driving separation in relation to HPFs at 6 mo, and two bacterial phylotypes (Porphyromonadaceae and Clostridiaceae) were driving the separation of the gut microbiome composition for all three diets at 16 mo. Taken together, this suggested that host age was a major driver of microbiome composition independent of diet. This was further supported by a weaker separation of the gut microbiomes between HFS and HFP with host age as shown in Fig. 7.

Overall, the most pronounced changes in the composition of the gut microbiota were observed in relation to age (Fig. 7) and dietary fat content (Fig. 5). Although gut microbiomes separated in response to changes in the dietary protein/sucrose ratio (Fig. 6), this was not as prominent as the impact of a high-fat diet.

DISCUSSION

Experiments using rodent models of high-fat diet-induced obesity have provided considerable insight into obesity-related metabolic disorders (20). However, by using such models it is difficult to distinguish obesity-related effects from effects elicited by high-fat feeding. To discriminate between these factors, we took advantage of the observation that obesity is prevented if the increase in dietary fat is accompanied by an increased protein/sucrose ratio (12, 14, 30, 32, 33, 36). Short-term effects of high-protein diets are well described (12, 14, 30, 32, 33, 36), but less is known in relation to long-term effects. Therefore, we fed mice high-fat diets with both a high and a low protein/sucrose ratio throughout life to investigate the long-term effects on metabolism and survival.

As previously demonstrated in short-term studies with male mice (14, 30, 32), high-protein feeding protected mice from developing high-fat diet-induced obesity. This study demonstrates that high-protein feeding has a persistent long-term effect preventing high-fat diet-induced obesity. The lean phenotype of the HFP-fed mice, together with the obese HFS-fed mice, provides a unique opportunity to distinguish the impact of high-fat feeding from that of obesity development in relation to survival and gut microbiota composition.

The mice fed a HFS diet exhibited a significant reduction in survival compared with the REF-fed mice. Moreover, the mice fed a high-protein diet were protected from a significant reduction in survival compared with the REF-fed mice, in agreement with a previous study (22), demonstrating that an increase in the protein/sucrose ratio protects mice against high-fat diet-induced reduction in survival. In line with our observation that an initially increased caloric intake and expansion of adipose tissue were associated with a reduced survival in HFS-fed mice, Keipert et al. (22) concluded that the harmful effects of high-fat diets on longevity were linked to early and rapid obesity development. Thus, the results from their study and our study indicate that the state of obesity, rather than high-fat feeding, is associated with reduced survival. In contrast to these studies, the use of a matrix-based approach for changing the macronutrient composition of the feed concluded that lifespan increased with a decrease in the protein/carbohydrate ratio (43). It is possible that the discrepancies between our study and that of Solon-Biet et al. in relation to the effect of protein/carbohydrate ratio on lifespan may be due to the fact that we used a higher protein/carbohydrate ratio, mainly due to a lower carbohydrate content in our HFP diet. Nevertheless, in agreement with our study, a low protein/carbohydrate ratio was also associated with increased body fat and hepatic lipid accumulation.

The timing of high protein intake in humans has been considered in relation to lifespan, where a high intake of animal proteins during middle age (50–65 yr) was reported to be associated with an increase in all-cause mortality linked to...
a higher level of IGF-I, whereas decreased mortality was reported in older persons (above 66 yr) (25). However, a high protein intake in this study was defined as 20 energy% or more and a low protein intake as less than 10 energy%, while all the diets from our mouse experiment had a protein content close to or above the definition for high protein intake in the human study. The impact of IGF-I, in addition to insulin signaling, has also been related in earlier studies to lifespan (2), where an extended lifespan and reduced fat mass have been demonstrated in mice with disruption of the insulin receptor in adipose tissue (3). In earlier short-term studies, the insulin level in HFP-fed mice was demonstrated to be significantly reduced.

Fig. 5. Effects of diet on the composition of the gut microbiota. Redundancy analysis, RDA, of microbiota composition in fecal samples collected from cages at 3 mo (A), 6 mo (B), and 16 mo (C) of feeding. Animal weight (red arrow) and the different diets [REF (black), HFS (red), HFP (blue)] were included as explanatory variables. These variables explain 42.6% (A), 45.9% (B), and 44.4% (C) of total variation. Relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best-fitting 15 genus groups are shown in the plots.
compared with a HFS-diet (30, 32). Thus, a reduction in insulin levels by a HFP diet and extended lifespan with reduced insulin signaling and fat mass are thereby in line with the protection from a high-fat diet-induced increase in mortality of the HFP-fed mice in our study.

An impact of sex on lifespan has been demonstrated in several studies (29, 39), where an extended lifespan is generally reported in females compared with males. Since IGF-I has been linked to survival, it is of interest that there are reported sex differences in lifespan of mice with heterozygous knockout of the IGF-I receptor (21). In the study of Solon-Biet et al. (43) using both male and female mice, no differences in lifespan in relation to diet were observed, whereas sex-specific differences in reproductive function were observed (44).

Female mice are largely protected against metabolic disorders despite obesity and increased serum triacylglycerol and cholesterol when fed a high-fat diet (35). However, similarly to our earlier findings in male mice, high-fat feeding in combination with high amounts of sucrose leads to impaired glucose tolerance (14, 30). A high protein/sucrose ratio did not protect

Fig. 6. Comparison of gut microbiota between mice fed HFP and HFS diets. RDA of microbiota composition in fecal samples collected from cages at 3 mo (A), 6 mo (B), and 16 mo (C) of feeding the HFS and HFP diets. Explanatory variables are weight of the animals (red arrow) and HFP (blue) and HFS (red) diets. These variables explain 19.7% (A), 25.5% (B), and 26.6% (C) of total variation. Relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best-fitting genus groups are shown in the plots.
the mice against high-fat diet-induced impaired glucose tolerance when mice were fed for 3 mo despite protection against obesity. Supporting this, expression of \textit{Pepck}, encoding the rate-limiting enzyme in hepatic gluconeogenesis, was increased in HFP-fed mice. In agreement with a similar report of a transiently impaired glucose homeostasis in high-fat/high-protein-fed mice (22), we observed no difference in glucose clearance between the groups after 18 mo.

A high protein/sucrose ratio protected the mice against high-fat diet-induced accumulation of triacylglycerol in the...

Fig. 7. Effect of age on composition of gut microbiota. RDA of microbiota composition in fecal samples collected from the cages \((n = 3–7\) cages per diet) at 3, 6, and 16 mo of feeding REF (A), HFS (B), or HFP (C) diet. Explanatory variables are weight of animals and time points. These variables explain 39.1% (A), 45.3% (B), and 47.9% (C) of total variation. Relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best-fitting 15 genus-like groups are shown in the plots.
liver for at least 6 mo. Moreover, the high protein/sucrose ratio alleviated the initial high-fat diet-induced expression of inflammatory markers in adipose tissue. In line with an earlier report that female mice were protected against high-fat diet-induced macrophage infiltration (35), we did not detect increased expression of macrophage-selective markers in samples obtained after 3 and 6 mo of feeding. However, after 18 mo of feeding, expression of the macrophage infiltration markers Cd68 and Emr1 in adipose tissue was surprisingly higher in the HFP-fed mice than in the HFS- and REF-fed mice.

DGE analysis in adipose tissue and liver demonstrated different patterns of expression over time in response to the dietary protein/sucrose ratio. Further RT-qPCR analyses of liver mRNA indicated that a high protein/sucrose ratio led to increased amino acid catabolism and ureagenesis as well as reduced lipid synthesis during the first month of feeding, in agreement with our earlier short-term experiments in male mice (14, 30, 32). Our study also demonstrated that the increased expression of genes linked to amino acid degradation and gluconeogenesis was maintained in the HFP-fed mice after 18 mo of feeding. By contrast, the changes in expression of genes involved in fatty acid synthesis were not sustained throughout the study.

As the gut microbiome is modulated in response to diet (6, 26), obesity (1, 8, 40, 47, 48), inflammation (5), and host age (46, 52), it was of interest to follow how the different diets affected the gut microbiota over time. In agreement with previous reports (6, 51), we observed that dietary fat was a strong driver of the composition of the gut microbiota. Moreover, we found that the protein/sucrose ratio significantly affected microbiome composition. The dietary protein/sucrose ratio regulates the development of obesity in mice (30), and an obese phenotype may in itself impact the microbiota (26).

However, we observed an increasing difference in obesity over time between HFS- and HFP-fed mice, which occurred in parallel with a decrease in differences between the gut microorganisms of these groups, suggesting that obesity-independent changes in the gut microbiome occurred in response to aging and dietary protein/sucrose ratio. Thus, a distinct microbiome composition was observed at 3, 6, and 16 mo of age within all three dietary groups, confirming that age-dependent changes occurred in the gut microbiome. Interestingly, although the bacterial phylotypes driving the longitudinal differences within each dietary group were not the same for the three diets at 3 mo, the interdietary differences diminished with time, suggesting that host age is a major driver of the gut microbiome composition independently of diet.

Age-related changes reported in elderly people (7) may be associated with decreased capacity for energy harvest from the food (48). In our study, Akkermansia muciniphila, a bacterium previously associated with leanness and inversely correlated with body weight (9), was driving the difference in gut microbiome composition at 6 mo for animals fed the two high-fat diets. Interestingly, at that time point the HFS-fed mice, but not the HFP-fed mice, were significantly more obese than the REF-fed mice, suggesting that Akkermansia was affected by dietary fat rather than by bodyweight.

Overall, high dietary fat content and host age, independent of obesity, appeared to be the two most important drivers of gut microbiota composition in this study. In contrast to this, the survival of the mice was significantly reduced in the obese HFS-fed mice compared with the low-fat REF group but not in the lean HFP-fed mice, suggesting obesity to be more important for survival than the high dietary fat intake. This is also in line with the changes related to progression of colon cancer (28), reported to be induced by high-fat feeding but resulting from obesity and not diet. These results highlight the importance of separating the impact of high-fat feeding from that of obesity. In the present study, dietary fat content was a more important driver of the gut microbiota composition than obesity, as the mice fed the HFS and HFP diets had similar gut microbiota profiles despite differences in obesity development.

In conclusion, our findings support the notion that reduced survival in response to high-fat feeding is linked to obesity development in mice fed a diet with a low protein/sucrose ratio. A high protein/sucrose ratio in the diet protected against high-fat diet-induced obesity and hepatic lipid accumulation and a significant reduction in survival. We observed the greatest diet-dependent differences in gene expression during the early period of growth. Over time, these differences diminished, pointing to the importance of the early growth period. We detected marked effects on the composition of the gut microbiota over time, and fat content in the diet and age, rather than adiposity or protein/sucrose ratio, were observed to be major drivers shaping the gut microbiota.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


