BASIC—ALIMENTARY TRACT

High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity

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BACKGROUND & AIMS: The composition of the gut microbiome is affected by host phenotype, genotype, immune function, and diet. Here, we used the phenotype of RELMβ knockout (KO) mice to assess the influence of these factors. **METHODS:** Both wild-type and RELMβ KO mice were lean on a standard chow diet, but, upon switching to a high-fat diet, wild-type mice became obese, whereas RELMβ KO mice remained comparatively lean. To investigate the influence of diet, genotype, and obesity on microbiome composition, we used deep sequencing to characterize 25,790 16S rDNA sequences from uncultured bacterial communities from both genotypes on both diets. **RESULTS:** We found large alterations associated with switching to the high-fat diet, including a decrease in *Bacteroidetes* and an increase in both *Firmicutes* and *Proteobacteria*. This was seen for both genotypes (ie, in the presence and absence of obesity), indicating that the high-fat diet itself, and not the obese state, mainly accounted for the observed changes in the gut microbiota. The RELMβ genotype also modestly influenced microbiome composition independently of diet. Metagenomic analysis of 537,604 sequence reads documented extensive changes in gene content because of a high-fat diet, including an increase in transporters and 2-component sensor responders as well as a general decrease in metabolic genes. Unexpectedly, we found a substantial amount of murine DNA in our samples that increased in proportion on a high-fat diet. **CONCLUSIONS: These results demonstrate the importance of diet as a determinant of gut microbiome composition and suggest the need to control for dietary variation when evaluating the composition of the human gut microbiome.**

The prevalence of diet-induced obesity is reaching epidemic proportions in industrialized nations. In parallel, there has been a dramatic increase in type 2 diabetes mellitus.[1] Together, these 2 diseases are an enormous health and financial burden. To maintain body mass, energy input through food intake and absorption must match energy expenditure through physical activity, basal metabolism, and adaptive thermogenesis. Because triglycerides, stored as fat in white adipose tissue, are the most efficient means of energy storage, alterations in energy balance favoring "energy input" can lead to obesity.[2]

Growing evidence demonstrates that the normal gut microbiome contributes to the development of diet-induced obesity.[3–5] The human intestine is home to some 100 trillion microorganisms, representing hundreds and perhaps thousands of species. The density of bacterial cells in the colon has been estimated at 10^{11} to 10^{12} per milliliter, which makes the colon one of the most densely populated microbial habitats known on earth.[6,7] The genome size of this pool of intestinal microbes is estimated to exceed the size of the human nuclear genome by 2 orders of magnitude.[7]

The colonization of germfree mice with the normal gut microbiota harvested from conventionally housed mice leads to an increase in fat mass despite a decrease in food intake.[8] Furthermore, the gut microbiome of ob/ob mice, which are hyperphagic and become morbidly obese because of the absence of the leptin satiety factor, exhibits an altered ratio of abundance in the 2 dominant bacterial divisions, the *Bacteroidetes* and the *Firmicutes*,[3] similar to that observed in obese humans.[4] Metagenomic analysis of these communities reveals an increased representation of genes encoding proteins important for the synthesis of short-chain fatty acids from the fermentation of dietary carbohydrates,[3] which can be utilized by the host for hepatic lipogenesis.[8] In this manner, the microbiome

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associated with obesity demonstrates an increased capacity to salvage energy from the diet.

We have previously reported the characterization of a colonic goblet cell-specific gene, RELMβ, whose expression is dependent on the presence of the gut microbiome.[9] T helper cell (Th) 2-mediated immune responses strongly activate the expression of RELMβ.[10] In turn, RELMβ has been shown to be an effector of intestinal immune function.[11–13] RELMβ protein is secreted apically into the lumen of the bowel by goblet cells, is found at high levels in the stool, and can also be detected in the serum.[9,14] A high-fat diet induces the expression of RELMβ in the stool.[14]

Herein, we take advantage of the RELMβ phenotype to investigate the linkage between diet, obesity, and gut microbiome composition. We used deep sequencing of 16S rDNA gene segments and shotgun metagenomic analysis of fecal bacteria to show that both RELMβ and diet, but not the metabolic phenotype of the host, are independent determinants of the gut microbiome.

Materials and Methods

Animals

RELMβ KO mice were generated on a mixed background, 129Svev/C57BL/6, as previously described.[11] Female RELMβ KO mice were back bred for 5 generations onto a C57BL/6 background. Wild-type and KO mice were generated from heterozygote parental crosses and were maintained on standard chow (LabDiet 5001; LabDiet, St Louis, MO) or high-fat diet (Research Diets D12451; Research Diets, Inc, New Brunswick, NJ). The composition of these 2 diets is shown in Supplementary Table 1. Food intake was assessed over 24 hours for a minimum of 3 consecutive days. All mice had free access to chow and water and were maintained on a 12-hour light cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

We have studied the effect of a high-fat diet on the development of diet-induced obesity in several cohorts of RELMβ KO and wild-type mice on a 129Svev/C57BL/6 background[11] as well as additional cohorts of mice backcrossed 5 generations to a C57BL/6 strain. Although a divergence in weight was observed in both sexes of 129Svev/C57BL6 background mice, this effect was observed only in female mice on the C57BL/6 background. Sexual dimorphic effects on diet-induced obesity have been previously described in mice.[15] Four of these studies showed the divergence of weight on a high-fat diet described above, and 2 studies failed to reproduce these findings, indicating incomplete penetrance of the phenotype.

Antibiotic Treatment of Mice

Twenty female C57BL/6 mice 14 weeks of age were equally divided into 4 groups: standard chow, standard chow with oral antibiotics, high-fat diet, and high-fat diet with oral antibiotics. The oral antibiotics, which were delivered in drinking water, consisted of ampicilllin (1.0 g/L), neomycin sulfate (1.0 g/L), metronidazole (1.0 g/L), and vancomycin (0.5 g/L) as previously described.[16] After 1 month, spontaneously voided fecal pellets were collected, and the colonic tissue was harvested for RNA isolation.

Measurement of Dietary Fat Absorption

Fats with negligible amounts of behenic acid (eg, safflower oil) were fed to mice along with the sucrose behenate esters (5% of the total dietary fat). After 2 days of ad libitum ingestion of the diet, approximately 10–30 mg of a fecal sample was analyzed by gas chromatography (GC) for fatty acids following saponification and methylation.[17]

Assessment of Body Composition

Magnetic resonance imaging (MRI) was performed using Echo MRI 3-in-1, with fat and lean magnetic resonance analysis (Echo Medical Systems, Houston, TX).

Indirect Calorimetry

Energy expenditure was measured by open-circuit calorimetry (Oxymax System; Columbus Instruments, Columbus, OH), and locomotor activity was measured simultaneously by infrared beam interruption (Optovarimax System; Columbus Instruments). Mice were housed individually in calorimetry cages at approximately 22°C and acclimatized for 24 hours. Room air was pumped at a rate of 0.52 L/min, and exhaust air was sampled at 27-minute intervals for 24 hours.

Core Body Temperature

Rectal body temperatures were measured at room temperature using a thermistor (YSI Model 4600; YSI Temperature, Dayton, OH).

RNA Isolation, Quantitative Reverse-Transcription Polymerase Chain Reaction, and RELMβ Immunoblot

RNA was isolated followed by reverse transcription and Syber Green quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for RELMβ and GAPDH as previously described.[10] The method used for the isolation of stool protein and immunoblot detection using a polyclonal donkey anti-rabbit antibody to mRELMβ has been previously described.[9]

Transitions in Gut Bacterial Populations Associated With Transition From Standard Chow to High-Fat Diet

Five female RELMβ KO mice and 5 female wild-type littermate controls were raised on standard chow (LabDiet 5001) for 13 weeks, at which time fecal pellets

were collected and stored at −80°C for subsequent DNA isolation and protein extraction. Mice were then switched to a high-fat diet (Research Diets D12451) for 21 weeks, at which time fecal pellets were again collected, stored at −80°C, and used for DNA isolation and protein extraction. Previous studies have documented that pellets contain bacterial populations resembling those present in the lower gastrointestinal (GI) tract and so provide a convenient sample source.[18] DNA was extracted and bacterial 16S rDNA composition quantified using the 454/ Roche GS FLX (454 Life Sciences, Branford, CT) as in McKenna et al,[19] and metagenomic analysis was carried out by shotgun sequencing of pooled DNA from pellets according to the 454/Roche protocol (http:// www.454.com/). All sequences will be deposited in Gene-Bank upon acceptance of the paper for publication. We identified the lowest common taxonomic ancestor of the DNA sequences from BLAST hits (bit score, >50) on the nucleotide (nt) database using the MEGAN algorithm.[20] To study the functional genomics of the samples, we collected BLAST hits (e-value < 0.00001) to UniProt sequences annotated to cellular process pathways by the *Kyoto Encyclopedia for Genes and Genomes*[21] using annot8r (http://www.nematodes.org/bioinformatics/annot8r/ index.shtml). Gene function was further investigated using the metagenomics RAST Server (default settings).[22] Statistical analysis was carried out in R (statistical programming language).

Results

Experimental Plan

We sought to determine the changes in the gut microbiome associated with a high-fat diet in mice and to determine whether the obese state or the high-fat diet itself accounted for any changes observed. We took advantage of the phenotype of RELMβ KO, which in this cohort remained comparatively lean on the high-fat diet compared with the wild-type controls (this phenotype is incompletely penetrant but was quite strong in the cohort studied here). Mutant or wild-type mice were maintained on standard chow, and pellets were harvested. Next, mice were switched to the high-fat diet for 21 weeks and pellets sampled again. Below, we first describe the RELMβ KO phenotype in this cohort and then describe the associated microbiome on the 2 diets.

A High-Fat Diet Enhances the Expression of RELMβ Dependent on the Presence of the Gut Microbiome

We have previously shown that RELMβ expression[23] in the colon and stool[9] is dependent on microbial colonization of the intestinal tract. Alteration in diet also effects RELMβ expression. Mice on a high-fat diet and obese db/db mice lacking the leptin receptor have higher
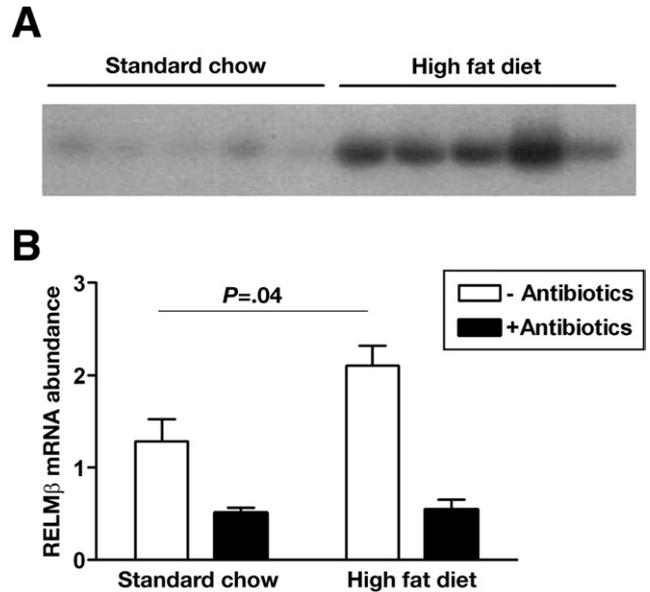

Figure 1. Induction of RELMβ expression in the stool and colon is dependent on gut bacteria. (*A*) RELMβ immunoblot using proteins isolated from fecal pellets collected from wild-type mice fed a standard chow diet for 13 weeks and again after 21 weeks on a high-fat diet. (*B*) Quantitative RT-PCR of colonic mRNA for RELMβ in mice fed a standard chow diet and a high-fat diet with and without the administration of oral antibiotics, mean ± SEM, n = 5 mice per group.

levels of colonic RELMβ expression.[14] Figure 1*A* shows that C57/B6J mice fed a high-fat diet for 3 months exhibited higher levels of RELMβ protein in the stool. The induction of RELMβ expression by a high-fat diet is dependent on commensal gut microbiota because treatment of mice with orally delivered antibiotics reduces the messenger RNA (mRNA) expression of colonic RELMβ similarly in both mice fed a standard chow as well as a high-fat diet (Figure 1*B*).

RELMβ KO Mice Remain Comparatively Lean on a High-Fat Diet

RELMβ KO mice do not exhibit an overt phenotype[11] (data not shown), but our cohort showed altered weight gain on the high-fat diet. Although RELMβ KO mice weighed the same as wild-type mice at 13 weeks of age on a standard chow diet, after 21 weeks on a high-fat diet, RELMβ KO mice exhibited diminished weight gain (Figure 2*A*) because of decreased accumulation of fat mass relative to wild-type controls (Figure 2*B*). The reduction in diet-induced obesity in these RELMβ KO mice was not due to an alteration in food intake, fat absorption, or core body temperature (Figures 2*C–E*) but was rather caused by an increase in energy expenditure, as measured by an increase in oxygen consumption over a period of 4 hours (light cycle) via indirect calorimetry (Figure 2*F*). Importantly, RELMβ KO did not exhibit any difference in physical activity compared with wild-type controls during this period (data not shown).
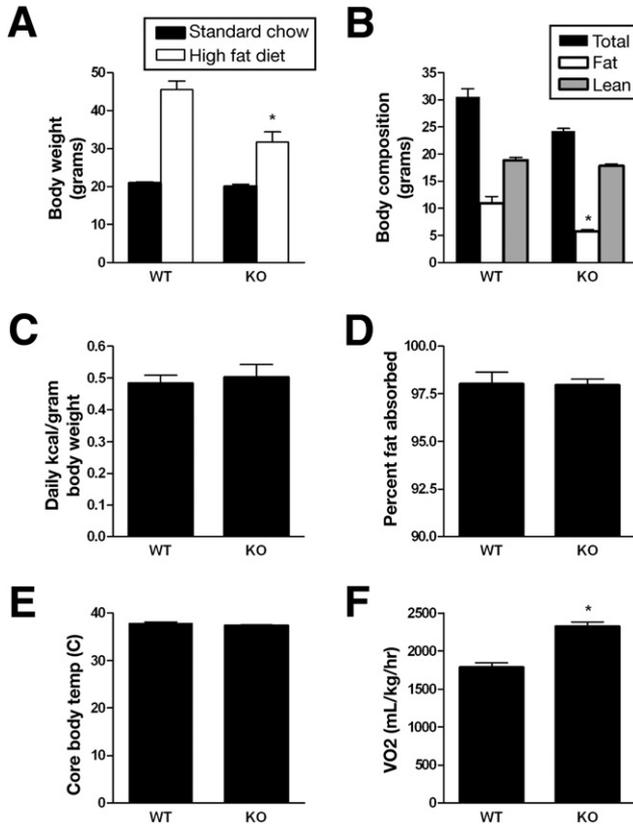
Figure 2. RELMβ KO mice remain comparatively lean on a high-fat diet compared with wild-type littermate controls. (*A*) Body weight of female RELMβ wild-type (WT) and knockout (KO) mice at 13 weeks of age on a standard chow diet or after 21 weeks on a high-fat diet, mean ± SEM, n = 4 or 5 mice per group; *P < .05. (*B*) MRI body composition analysis after 8 weeks on the high-fat diet, mean ± SEM, n = 4 or 5 mice per genotype, *P = .004. (*C*) Daily food intake in RELMβ KO and wild-type mice fed a high-fat diet for 4 weeks, mean ± SEM, n = 4 or 5 mice per genotype. (*D*) Percent dietary fat absorbed by RELMβ KO and wild-type mice fed a high-fat diet for 5 weeks, mean ± SEM, n = 4 or 5 mice per genotype. (*E*) Rectal temperatures of RELMβ KO and wild-type mice fed a high-fat diet for 8 weeks, mean ± SEM, n = 4 or 5 mice per genotype. (*F*) Oxygen consumption (VO₂) measured via indirect calorimetry over 4 hours (light cycle) in RELMβ KO and wild-type mice after 21 weeks on a high-fat diet, mean ± SEM, n = 4 mice per group, *P < .0001.

Transitions in Gut Bacterial Populations Associated With Diet

To determine the effect of a high-fat diet on the composition of the gut microbiome, spontaneously voided fecal pellets were collected from the 5 RELMβ KO mice and 5 wild-type controls at 13 weeks of age while on a standard chow diet and again after 21 weeks on a high-fat diet. DNA was purified from pellets, and samples were analyzed by 16S rDNA profiling and metagenomic analysis using 454/Roche Pyrosequencing.

The 16S rDNA PCR primers were chosen based on the published reconstruction studies of Liu et al to maximize the reliability of community analysis and phylogenetic assignments.[24] To analyze all the 16S rDNA sequences in

parallel, samples were amplified using bar coded primers as previously described[19,25] and individual samples sorted after sequencing using the bar code information. A total of 25,790 sequence reads passed quality filters with an
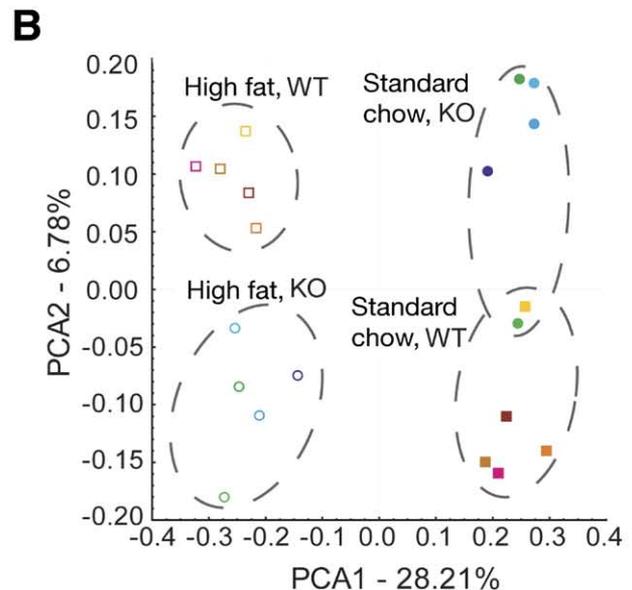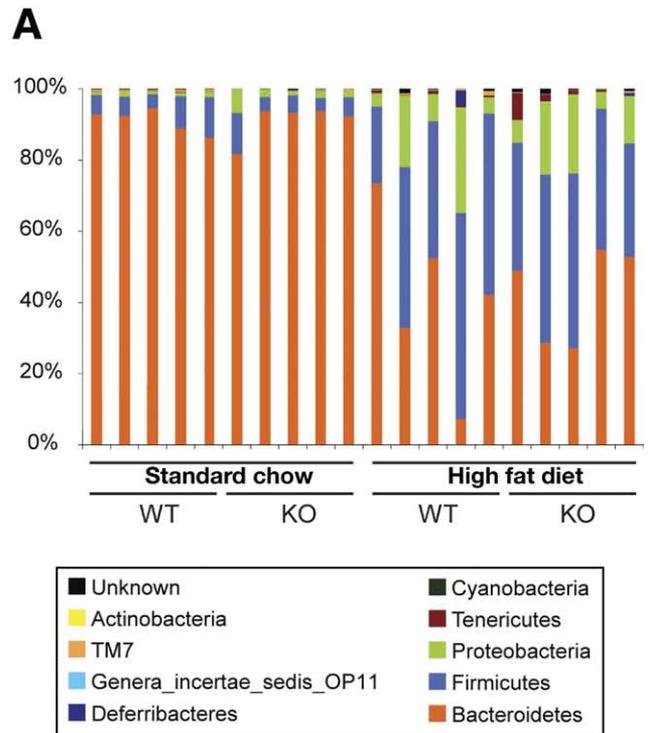
Figure 3. Analysis of gut bacterial communities by 16S rDNA analysis from mice on the standard chow and high-fat diets. (*A*) The Figure shows the percentages of each community contributed by the indicated phyla. Diet and genotype are indicated below the Figure. (*B*) UniFrac analysis of the bacterial communities studied. Each *point* corresponds to a community from a single mouse. Samples from the standard chow and high-fat diet are from the same mouse, as indicated by the color code. *Open symbols* indicate high-fat diet, *closed symbols* the standard chow. *Circles* indicate the knockouts; *squares* the wild-type controls. *Colors* indicated individual mice.

average read length of 262 nt. Sequence counts per sample ranged from 617 to 2448 per sample.

Sequence reads were aligned using NAST and the GreenGenes database, and phylogenetic placements were determined using ARB's parsimony insertion tool and the Hugenholtz tree.[26,27] Taxonomic assignments were then extracted from the phylogenetic tree (Figure 3A). Communities from both wild-type and RELMβ KO mice on standard chow diet were relatively similar in composition among the 10 samples. Each was dominated by gram-negative anaerobes of the *Bacteriodetes* phylum. The next most abundant group was *Firmicutes*, particularly of the anaerobic genus *Clostridia*. Less abundant but detectable were bacteria of the *Proteobacteria*, *Tenericutes*, and *TM7* phyla (Figure 3A).

Samples from the wild-type mice after 3 months on the high-fat diet resulted in a drastic change in the detectable 16S rDNA sequences (Figure 3A). In all 10 samples, the *Firmicutes* class *Clostridiales* was greatly expanded at the expense of the *Bacteroidetes* class *Bacteriodales*. The *Delta-Proteobacteria* were also greatly expanded. Within the *Bacteriodetes*, more than 30 different lineages were reduced in abundance. Orders affected included *Bacteriodaceae*, *Prevotellaceae*, and *Rickenellaceae*. The increase in *Firmicutes* was due to an increase in *Clostridiaceae*. The bloom of *Proteobacteria* was largely accounted for by the *Desulfovibrionaceae*. A previous study of high-fat diet in mice reported a bloom of *Mollicutes*,[5] and we saw an increase in *Mollicutes* as well, but, numerically, these were comparatively modest in number in our study. To confirm the taxonomic placements of the main lineages detected, 184 near full-length 16S rDNA sequences were determined from these communities. Analysis indicated that these yielded phylogenetic placements were consistent with the Pyrosequence data (Supplementary Table 2).

The High-Fat Diet, and Not the Obese State, Accounts for the Altered Microbial Communities

The changes seen between diets for the wild-type community could have been due either to the obese state resulting from the high-fat diet or to the direct effects of the diet on bacterial populations. The RELMβ KO mice remained comparatively lean on the high-fat diet, allowing us to distinguish between these models. As can be seen in Figure 3A, the general changes in the composition of the gut microbiome were similar between wild-type and KO mice, indicating that effects of diet dominated.

Figure 3B presents a global analysis of the communities based on the pair-wise phylogenetic distances calculated using UniFrac.[28,29] Pairs of communities were marked on a common phylogenetic tree, and then the fraction of the branch length unique to each community was determined. This provides a measure of the distance between communities in terms of their shared evolution-

ary history. Distances for all pairs of communities were than calculated, and principal coordinate analysis was used to generate the scatter plot in Figure 3B. The first principal coordinate (x-axis), which explained 28% of the variance, separated the communities by the type of diet. The second principal coordinate (y-axis), which accounted for 7% of the variance, separated the communities by genotype within each diet. The final position of the 2 genotypes differed within diets, indicating an interaction between diet and genotype. Thus, the difference in diet had the predominant effect on community structure, but effects of REMLβ genotype were detectable.

Bacterial Lineages Affected by RELMβ

Analysis of lineages indicated that much of the difference between RELMβ KO and wild-type mice was due to changes in abundance of relatively low-level lineages. Fifteen *Bacteriodetes* lineages, and 1 lineage of *Proteobacteria*, changed in abundance between genotypes, whereas 15 *Firmicutes* lineages changed in abundance. Although detectable, these changes were small compared with those associated with diet.

Metagenomic Analysis of Gut Bacterial Populations From Mice on Standard Chow or High-Fat Diets

To investigate the changes in bacterial gene content associated with diets in more detail, we carried out a metagenomic analysis of pellets from wild-type mice on the standard chow and high-fat diets. Bacterial cells are known to exchange DNA sequences by a wealth of mechanisms,[30] so the types of bacteria present, as inferred from 16S rDNA sequence reads, may not be reflective of the gene content of the bacterial communities. The DNA samples from the gut communities were combined from mice on either standard chow or mice on the high-fat diet, and then samples were sheared, ligated to linkers, and subjected to 454 Pyrosequencing. The average length sequence read returned was ~260 nt. A total of 239,905 sequences were recovered for the standard chow pool and 297,699 for the high-fat diet pool (Figure 4).

MEGAN was used to identify the taxonomic origin of the DNA sequences assignable with the BLAST cut-off used, revealing unexpected differences between the 2 diets (Figure 4A). Taxa were assigned for 41,980 of the standard chow reads and 79,257 reads from the high-fat diet. On the standard chow diet, 81% of the sequence reads were annotated to within the Bacteria domain and 19% to the Eukaryote domain. Of the eukaryotic reads, the vast majority was further classified as murine. In samples from the high-fat diet, the murine assignments were the most abundant, accounting for 61% of reads, whereas bacterial sequences only accounted for 39%. This was further supported by using a quantitative PCR assay for bacterial 16S rDNA sequences, which showed that the
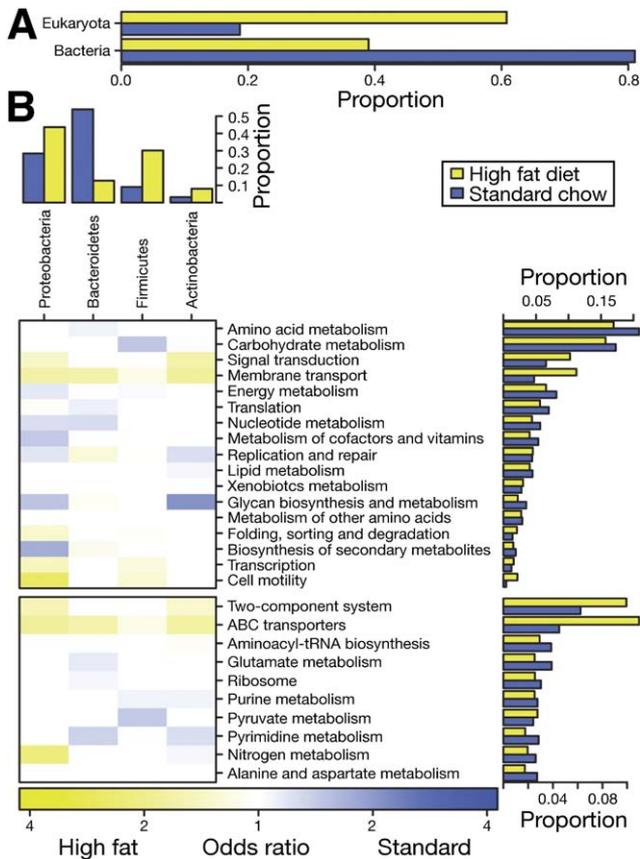
Figure 4. Metagenomic analysis of community composition. Samples were pooled for wild-type mice from standard chow (*blue*) and high-fat (*yellow*) diets. (*A*) Comparison of contributions of bacterial and murine DNA for the 2 communities. Groups were assigned using MEGAN. A great majority of sequences from "Eukaryota" were murine. (*B*) Metagenomic analysis of bacterial taxa and gene types. The bacterial phyla are indicated along the top of the Figure for the 4 most abundant bacterial phyla (95% of total), the functional categories in the *column* to the right. The *colored tiles* in the body of the Figure indicate the changes in gene content within each phylum (that is, changes in gene types are compared between diets considering only sequences from each phylum). Functional classes were assigned using KEGG and SEED annotation. The color scale (*bottom*) reports the odds ratio (set to the conservative edge of the confidence interval; if the interval included 1, the value was set to 1).

proportion of bacterial sequences was greater for the standard chow than the high-fat diet samples (data not shown). One possible explanation is that the high-fat diet led to greater shedding of intestinal epithelial cells into the lumen leading to the accumulation of mouse DNA in fecal pellets.

The bacterial phyla identified using MEGAN included *Proteobacteria*, *Bacteriodetes*, *Firmicutes*, and *Actinobacteria*, paralleling the 16S rDNA analysis. After the switch to the high-fat diet, the proportions of *Proteobacteria*, *Firmicutes*, and *Actinobacteria* increased and *Bacteriodetes* decreased, paralleling results with 16S rDNA sequencing. A few minor groups did differ between the metagenomic and 16S rDNA data. For example, the *Deferribacteres* was not

identified in the metagenomic data, probably because there are no full genome sequences for this group in the database to serve as a target for alignment.

Shotgun DNA sequences were aligned to the UniProt database using BLAST and assigned to functional categories using SEED and KEGG annotation. KEGG pathways were assigned for 33,267 reads from the standard chow reads and 33,465 from the high-fat diet.

The proportions of gene types were then compared between the 2 diets (Figure 4B, *column* to the right of the figure). Multiple categories showed changes in abundance, many of which are involved in metabolism. Genes for amino acid metabolism and carbohydrate metabolism decreased in abundance upon the switch to the high-fat diet, whereas genes for signal transduction and membrane transport increased. Finer level analysis showed that most of the genes involved in signal transduction were 2-component response-regulator systems, and the membrane transport molecules were mostly ABC transporters (Figure 4B, *bottom right*).

The changes in gene complement are color coded as a heat map in the table in Figure 4B to show the changes in gene representation within each of the bacterial phyla in the 2 diets. Thus, within the *Proteobacteria*, lineages with more genes for signal transduction, cell motility, and membrane transport increased in abundance on the high-fat diet, whereas lineages with more genes for energy metabolism and nt metabolism decreased. For *Bacteriodetes*, lineages rich in genes for amino acid metabolism, translation, and nt metabolism decreased, whereas those with more genes for membrane transport and replication and repair increased. For the *Firmicutes*, lineages with more genes for membrane transport, transcription, and cell motility increased in abundance on the high-fat diet, whereas those with more genes for carbohydrate metabolism and energy metabolism decreased. Collectively, these data document a community-wide change in metabolism that was distinctive for each phylum accompanying the switch to the high-fat diet.

Under the high-fat diet, a collection of genes for ABC transporters increased in abundance. Analysis indicated that these transported a variety of nutrients including lipids, sugars, and peptides and metals. This may be a reflection of the different nutrients available in the high-fat diet or reduced precursor synthesis so that lineages with increased numbers of transporters were favored for growth. ABC transporters that act as efflux pumps for antibiotics were also increased in abundance in the high-fat diet, suggesting the possibility that high-fat diet may diminish the sensitivity of some bacterial groups to antibiotics.[31]

A collection of genes involved in import and assimilation of sugars were more abundant in samples from the high-fat diet, paralleling data of Turnbaugh et al.[5] Genes for utilization of fructose, raffinose, D-ribose, sucrose, and mannitol all increased in abundance. Genes for phos-

phorous metabolism were also increased on the high-fat diet, notably phosphotransferase systems active during the uptake and assimilation of sugars.

Several classes of genes changed in abundance as expected from the reduction in gram-negative *Bacteriodetes* on the high-fat diet. Genes responsible for synthesis of the gram-negative cell wall were decreased in abundance in the high-fat diet, and genes for gram-positive cell wall synthesis increased. Sequences matching a *Bacteriodetes* conjugative transposon declined sharply in abundance. Genes for respiration increased in the high-fat diet with the decline in anaerobic *Bacteriodetes*. A collection of genes involved in bacterial motility increased in abundance on the high-fat diet, and these were associated with the expanded *Proteobacteria* and *Firmicutes* phyla, indicating that the more motile members of these lineages expanded preferentially.

Our findings differed from those of Turnbaugh et al[5] regarding the relationship of bacterial motility in the 2 diets. We found that a collection of gene groups linked to bacterial motility increased on the high-fat diet, including "flagellar motility" and "motility and chemotaxis." In contrast, Turnbaugh et al found that categories related to flagellar assembly, motility, and chemotaxis decreased in abundance on a Western (high fat) diet. In summary, the metagenomic analysis documented a community-wide transition in gene content that accompanied the switch to the high-fat diet and specified gene classes affected within each lineage.

Discussion

We find, as have others,[4,5] that distinctive community-wide changes in the gut microbiome accompany a switch from a standard chow to a high-fat diet. This could be a result of either (1) the obese state altering the gut microbiome or (2) the high-fat diet causing changes in the microbiome directly. Here, we distinguished between these 2 models by taking advantage of the RELMβ KO phenotype. The RELMβ KO mice remained comparatively lean when subjected to the high-fat diet in our cohort, thereby allowing us to assay the effects of the high-fat diet in the absence of obesity. We found that consistent and dramatic changes in microbial communities could be seen upon switching to the high-fat diet for both wild-type and RELMβ KO mice, establishing that the high-fat diet itself, and not the obese state, was responsible for the altered microbiota.

In the cohort studied here, we found that RELMβ KO reduces diet-induced obesity without altering food intake or fat absorption by increasing energy expenditure. This occurred only on a high-fat diet. This finding is similar to other murine model systems in which alterations in energy expenditure have been shown to alter the development of obesity only on a high-fat diet.[32,33] Thus, our studies further confirm that relatively modest alterations

in energy expenditure can lead to dramatic differences in the accumulation of fat mass over time.

We initially hypothesized that RELMβ might regulate the composition of the gut microbiome because (1) RELMβ is expressed at high levels in the stool of both mice and humans[9] where it is dependent on the presence of the gut microbiome[9,23] and (2) other immune effector molecules secreted by the intestinal mucosa[34] (and also intestinal inflammation[35]) lead to alterations in gut microbiome composition. We and others have previously shown that RELMβ can function as an activator of innate immune cell populations where it can regulate intestinal inflammation.[11–13] Here, we report that RELMβ has a modest but significant effect on the composition of the gut microbiome, thereby specifying another host cell gene that regulates the microbiome.

Our results differ from those of Turnbaugh et al[5] in several ways. Rather than observing a bloom of *Mollicutes* on a high-fat diet, we observed a bloom of *Clostridia* and *Proteobacteria* (Figure 3). A major group of *Proteobacteria* that increased in abundance were the phylum *Delta-Proteobacteria*, order *Desulfovibrio*. This group contains sulfate-reducing bacteria, and genes for sulfate reduction were also detected as increased on high fat in the metagenomic analysis. However, even within *Bacteriodetes*, the lower level groups present differed considerably between the 2 diets, emphasizing the extent of the community changes between diets. The reason for the differences between our data and those of Turnbaugh et al are unclear: the sample types analyzed differed, and it is also possible that the starting gut microbiota in mice from the 2 groups differed so that different bacterial lineages bloomed upon switching to the high-fat diet.

The metagenomic analysis showed, unexpectedly, that the high-fat diet led to an increase in the proportion of murine DNA in pellets. It is estimated that, in humans, the self-renewal process of the intestinal epithelium results in the release of over 10^{10} cells into the gut lumen each day.[36] The proportional increase of murine DNA on a high-fat diet may, therefore, be a result of the enhanced intestinal epithelial proliferative response.[37] We note that another possible explanation for the data is that the absolute amount of bacteria was diminished in the gut on the high-fat diet: if the amount of murine DNA stayed the same, it would have increased as a proportion. Going forward, it will be of great interest to investigate the generality of this observation in other models and in humans, to assess whether the level of intestinal epithelial shedding is increased, and determine whether this can be linked to pathogenic effects of the high-fat diet.

The reduction of carbohydrates in the high-fat diet may have resulted in a state of nutrient stress on the gut microbiome. This notion is supported by the general decrease in a broad number of metabolic genes under the high-fat condition, a response to nutrient deficiency that has been observed in vitro.[38] Notable exceptions to this

trend are the high-fat induced ABC transporters, genes of the 2-component system, and genes associated with movement. Bacterial ABC transporters, which are ATP-dependent transmembrane proteins that play a role in the import of nutrient substrates,[31] may be induced to enhance nutrient uptake in an environment of limiting substrates. Genes for ABC transporters are enriched by a high-fat diet among the most predominant bacterial taxa in the gut microbiome. Two-component systems are the principal signal transduction mechanisms in bacteria allowing prokaryotic organisms to adapt to new environmental conditions under selective pressure.[39] Therefore, bacteria that have an enriched representation of this system may be better suited for adaptation to gut environment on a high-fat diet. Indeed, a 2-component system that couples glycan sensing to carbohydrate metabolism has been shown to be an important adaptation of *Bacteroides thetaiotaomicron*, a major gut commensal.[40] Finally, a high-fat diet enhances the representation of genes for bacterial chemotaxis and flagellar assembly. These genes are principally associated with the bloom of *Proteobacteria*, suggesting that bacterial movement may provide bacteria within this phylum a growth advantage.

There are several limitations of our study. First, we did not examine a separate cohort of mice that would serve as controls to evaluate the effect of aging on the composition of the gut microbiome. The effect of aging on the composition of the adult gut microbiome has not been extensively characterized. However, in human adults, the gut microbiome can remain relatively stable over months,[41,42] although variations with changes in diet can be extreme. Together with the similarities of our results with those previously reported to be associated with diet-induced obesity,[5] we believe that the impact of aging on our results would be comparatively minor. Second, we only measured the effect of an altered diet, so the importance of the different components is unknown. The amount of fat in the high-fat diet is increased by approximately 4-fold, whereas the amount of carbohydrate is reduced by less than 2-fold compared with the normal chow diet. Protein composition in the 2 diets remained roughly the same. We suggest that it is most likely that the increase in fat is the primary determinant of the observed effects because work of the Gordon laboratory showed that a high-fat and high-carbohydrate diet leads to similar alterations in the composition of the gut microbiome.[5] Third, it is important to note that metagenomic studies report only proportional differences in gene content, not absolute values. In addition, it is currently unknown to what degree the alterations of bacterial gene representation, identified by metagenomics, correlate with either RNA abundance, protein expression, or biologic function. Finally, additional studies with larger cohorts of mice will be required to characterize fully the effects of RELMβ on host metabolism.

Our results suggest that the substantial intersubject variability in the composition of the human gut microbome[18,43] may be due, in part, to variation in diet. This notion is supported by the relatively modest amount of variability in the composition of the gut microbiome in our study of mice, which are fed a defined diet, compared with a large amount of microbiome variability reported in macaques, which consume a much more varied diet.[19] Unless human studies are performed on subjects consuming a defined diet or methods are developed to adjust for dietary variation, it may be challenging if not impossible to make definitive associations between disease state in humans and alterations in the composition of the gut microbiome.[35,43]

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.08.042.

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BASIC–
ALIMENTARY TRACT

Supplementary Table 1. Comparison of the Composition of the Standard Chow and High-Fat Diets

	Standard chow, kcal %	High-fat diet, kcal %
Protein	28	20
Carbohydrate	60	35
Fat	12	45

NOTE. Values depicted for each component are percentage of total kilocalories (kcal %).

Supplementary Table 2. Assignment to the Genus Level for 184 Near Full-length 16S rDNA Sequences

Phylum	Class	Order	Family	Genus	WT normal	WT high fat	KO normal	KO high fat
Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes	*Anaerophaga*		1	1	1
				Bacteroides	2		2	
			Porphyromonadaceae	*Parabacteroides*	1		4	1
				Proteiniphilum	2			
				Tannerella	30	2	23	7
			Prevotellaceae	*Prevotella*	2		1	
			Rikenellaceae	*Alistipes*	4		1	
				Rikenella		1		1
Deferribacteres	Deferribacteres	Deferribacterales	*Deferribacteraceae*	*Mucispirillum*		3		
Firmicutes	"Clostridia"	Clostridiales	"Lachnospiraceae"	"*Lachnospiraceae Incertae Sedis*"		16	2	2
				Anaerostipes		5		
				Bryantella	2		1	3
				Butyrivibrio				1
				Catonella				1
				Johnsonella		1	1	
				Parasporobacterium	1	1	1	5
				Sporobacterium				1
			"Ruminococcaceae"	"*Ruminococcaceae Incertae Sedis*"		2	1	1
				Acetanaerobacterium		1	1	4
				Anaerotruncus			1	1
				Papillibacter				2
				Gallionella	1			
				Bilophila		12	5	11
Proteobacteria	Betaproteobacteria	Nitrosomonadales	Gallionellaceae	*Desulfovibrio*				1
	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Lawsonia*		1		1
	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	*Helicobacter*		1	1	2
				Sum	45	47	46	46
								84

NOTE. Assignments from the near full-length sequences closely matched the much larger number from the 454 Pyrosequence analysis. Taxonomic assignments were generated using RDP annotation.