



Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes

Gary D. Wu *et al.*

Science **334**, 105 (2011);

DOI: 10.1126/science.1208344

This copy is for your personal, non-commercial use only.

If you wish to distribute this article to others, you can order high-quality copies for your colleagues, clients, or customers by [clicking here](#).

Permission to republish or repurpose articles or portions of articles can be obtained by following the guidelines [here](#).

The following resources related to this article are available online at www.sciencemag.org (this information is current as of April 17, 2013):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:

<http://www.sciencemag.org/content/334/6052/105.full.html>

Supporting Online Material can be found at:

<http://www.sciencemag.org/content/suppl/2011/09/01/science.1208344.DC1.html>

A list of selected additional articles on the Science Web sites **related to this article** can be found at:

<http://www.sciencemag.org/content/334/6052/105.full.html#related>

This article **cites 12 articles**, 4 of which can be accessed free:

<http://www.sciencemag.org/content/334/6052/105.full.html#ref-list-1>

This article has been **cited by 38 articles** hosted by HighWire Press; see:

<http://www.sciencemag.org/content/334/6052/105.full.html#related-urls>

This article appears in the following **subject collections**:

Microbiology

<http://www.sciencemag.org/cgi/collection/microbio>

α -GalCer or through the blockade of noradrenergic neurotransmitters was sufficient to modulate iNKT cells in a manner that results in reduced infection and associated lung injury after stroke.

iNKT cells are emerging as an important population of cells crucial for the regulation of immunity. We have described an essential role of the sympathetic nervous system and iNKT cells in the defense against infectious complications after stroke. Although aspiration pneumonia is a contributing factor to increased infection in stroke patients, it cannot explain the immunosuppression noted by us and others. Our data suggest that a functional innervation of iNKT cells in the liver contributes to this immunosuppression. Our study also provides insights into the crosstalk that occurs between the central nervous system and the immune system, which is only beginning to be understood, and may be a step toward the development of an effective therapy for the number one killer in stroke patients, namely, infection. Although antibiotics may be a viable option for treatment, with the ever-increasing problem of antibiotic resistance, immunomodulation is an attractive alternative.

References and Notes

1. K. Kimura, K. Minematsu, S. Kazui, T. Yamaguchi; Japan Multicenter Stroke Investigators' Collaboration (J-MUSIC), *Cerebrovasc. Dis.* **19**, 171 (2005).
2. P. Langhorne *et al.*, *Stroke* **31**, 1223 (2000).

3. A. Chamorro, X. Urra, A. M. Planas, *Stroke* **38**, 1097 (2007).
4. C. Meisel *et al.*, *Stroke* **35**, 2 (2004).
5. A. Chamorro *et al.*, *J. Neurol. Neurosurg. Psychiatry* **77**, 1279 (2006).
6. R. J. Howard, R. L. Simmons, *Surg. Gynecol. Obstet.* **139**, 771 (1974).
7. L. Brossay *et al.*, *J. Exp. Med.* **188**, 1521 (1998).
8. T. Kawano *et al.*, *Science* **278**, 1626 (1997).
9. A. Bendelac, P. B. Savage, L. Teyton, *Annu. Rev. Immunol.* **25**, 297 (2007).
10. F. Geissmann *et al.*, *PLoS Biol.* **3**, e113 (2005).
11. P. Velázquez *et al.*, *J. Immunol.* **180**, 2024 (2008).
12. W. Y. Lee *et al.*, *Nat. Immunol.* **11**, 295 (2010).
13. H. Offner, A. A. Vandenbark, P. D. Hurn, *Neuroscience* **158**, 1098 (2009).
14. K. Prass *et al.*, *J. Exp. Med.* **198**, 725 (2003).
15. D. I. Godfrey, M. Kronenberg, *J. Clin. Invest.* **114**, 1379 (2004).
16. J. H. Lichtman *et al.*, *Neurology* **76**, 1976 (2011).
17. V. L. Roger *et al.*; American Heart Association Statistics Committee and Stroke Statistics Subcommittee, *Circulation* **123**, e18 (2011).
18. A. Liesz *et al.*, *Brain* **134**, 704 (2011).
19. H. Offner *et al.*, *J. Cereb. Blood Flow Metab.* **26**, 654 (2006).
20. H. Offner *et al.*, *J. Immunol.* **176**, 6523 (2006).
21. J. Mattner *et al.*, *Nature* **434**, 525 (2005).
22. B. McDonald *et al.*, *Science* **330**, 362 (2010).
23. M. Minagawa *et al.*, *Hepatology* **31**, 907 (2000).
24. H. Oya *et al.*, *Clin. Exp. Immunol.* **121**, 384 (2000).
25. L. Steinman, *Nat. Immunol.* **5**, 575 (2004).
26. K. J. Tracey, *Nat. Rev. Immunol.* **9**, 418 (2009).

Acknowledgments: We thank D. R. Littman (New York University School of Medicine) for the *Cxcr6^{flp/+}* knock-in mice, and the Live Cell Imaging Facility

funded by the Canada Foundation for Innovation and P. Colarusso for training and assistance related to microscopy. We thank C. Badick for excellent technical support. The NIH Tetramer Core Facility provided mouse PB557-loaded CD1d-tetramer for identification of iNKT cells by flow cytometry. We also thank the University of Calgary Flow Cytometry Facility and L. Kennedy for their assistance with the flow cytometric analysis. The work is supported by the Canadian Association of Gastroenterology (C.H.Y.W.), the Canadian Institutes of Health Research (C.H.Y.W., W-Y.L., and P.K.), the Canada Research Chairs Program (P.K.), the Alberta Innovates Health Solutions (C.N.J. and P.K.), and the Calvin, Phoebe, and Joan Snyder Chair for Translational Research in Critical Care Medicine (C.L.). The data reported in this paper are tabulated in the supporting online material. The authors declare no competing financial interests. C.H.Y.W. designed and did most of the experiments, analyzed the results, and prepared the manuscript; C.N.J. did some flow cytometry experiments; W-Y.L. isolated iNKT cells for the *in vitro* experiments; C.L. performed the multiplex mouse cytokine/chemokine assay; and P.K. provided overall supervision, helped design all of the experiments, and prepared the manuscript.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1210301/DC1
Materials and Methods
Figs. S1 to S13
References (27–32)
Movies S1 to S4

24 June 2011; accepted 4 August 2011
Published online 15 September 2011;
10.1126/science.1210301

Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes

Gary D. Wu,^{1*} Jun Chen,^{2,3} Christian Hoffmann,^{4,5} Kyle Bittinger,⁴ Ying-Yu Chen,¹ Sue A. Keilbaugh,¹ Meenakshi Bewtra,^{1,2} Dan Knights,⁶ William A. Walters,⁷ Rob Knight,^{8,9} Rohini Sinha,⁴ Erin Gilroy,² Kernika Gupta,¹⁰ Robert Baldassano,¹⁰ Lisa Nessel,² Hongzhe Li,^{2,3} Frederic D. Bushman,^{4*} James D. Lewis^{1,2,3*}

Diet strongly affects human health, partly by modulating gut microbiome composition. We used diet inventories and 16S rDNA sequencing to characterize fecal samples from 98 individuals. Fecal communities clustered into enterotypes distinguished primarily by levels of *Bacteroides* and *Prevotella*. Enterotypes were strongly associated with long-term diets, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*). A controlled-feeding study of 10 subjects showed that microbiome composition changed detectably within 24 hours of initiating a high-fat/low-fiber or low-fat/high-fiber diet, but that enterotype identity remained stable during the 10-day study. Thus, alternative enterotype states are associated with long-term diet.

We coexist with our gut microbiota as mutualists, but this relationship sometimes becomes pathological, as in obesity, diabetes, atherosclerosis, and inflammatory bowel diseases (1, 2). Factors including age, genetics, and diet may influence microbiome composition (3). Of these, diet is easiest to modify and presents the simplest route for therapeutic intervention. Recently, an analysis of gut microbial communities proposed three predominant variants, or “enterotypes,” dominated by *Bacteroides*, *Prevotella*, and *Ruminococcus*, respectively (4). The basis for enterotype clustering is

unknown but appears independent of nationality, sex, age, or body mass index (BMI).

Here, we investigated the association of dietary and environmental variables with the gut microbiota. First, in a cross-sectional analysis of 98 healthy volunteers (abbreviated “COMBO”), we collected diet information using two questionnaires that queried recent diet (“Recall”) and habitual long-term diet (food frequency questionnaire; “FFQ”). Second, 10 individuals were sequestered in a hospital environment in a controlled-feeding study (abbreviated “CAFE”) to compare high-fat/low-fiber and low-fat/high-fiber diets. Stool

samples were collected (5), and DNA samples were analyzed by 454/Roche pyrosequencing (6) of 16S rDNA gene segments and, for selected samples, shotgun metagenomics (7). In CAFE, rectal biopsy samples were also collected and analyzed on days 1 and 10.

For COMBO, we used 16S ribosomal DNA (rDNA) sequence information to calculate pairwise UniFrac distances (8) among the microbial communities. We assessed both relative abundance data (weighted analysis) and presence/absence information (unweighted analysis). Specific nutrients associated with variation in the gut microbiome for the 98 subjects were extracted, along with

¹Division of Gastroenterology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

²Center for Clinical Epidemiology and Biostatistics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

³Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

⁴Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

⁵Instituto de Ciências Biológicas, Universidade Federal de Goiás Goiânia, GO, 74001-970, Brazil.

⁶Department of Computer Science, University of Colorado, Boulder, CO 80309, USA.

⁷Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309, USA.

⁸Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309, USA.

⁹Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309, USA.

¹⁰Division of Gastroenterology, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA.

*To whom correspondence should be addressed. E-mail: gdwu@mail.med.upenn.edu (G.D.W.); lewisjd@mail.med.upenn.edu (J.D.L.); bushman@mail.med.upenn.edu (F.D.B.)

demographic factors (table S1). For each nutrient, we performed PERMANOVA (9) to test for nutrient microbiome association, from which we identified 72 and 97 microbiome-associated nutrients in Recall and FFQ, respectively, at a false discovery rate (FDR) of 25% (the relatively high value was used so as not to miss possible effects of diet on low-abundance bacteria). Both weighted and unweighted UniFrac identified similar nutrients, although the discrimination was sharper with unweighted UniFrac, indicating that change in community membership rather than community composition was the main factor.

For each of these nutrients, we used Spearman correlations to identify the associated bacterial genera. We considered only the 78 taxa that had abundance $\geq 0.2\%$ in at least one sample and appeared in more than 10% of the samples. Figure 1 shows a heat map summarizing Spearman correlations between nutrients from the FFQ and bacterial taxa. For a given taxon, individual nutrients account for 3 to 20% of the between-subject variation in abundance.

Nutrients of the same food groups from Recall and FFQ tended to cluster together (fig. S1A). The nutrients from fat versus plant products and fiber showed inverse associations with microbial taxa (Spearman $\rho = -0.68$, $P < 0.0001$). Inverse associations were also seen with amino acids and proteins versus carbohydrates (Spearman $\rho = -0.73$, $P < 0.0001$) and with fat versus carbohydrates (Spearman $\rho = -0.61$, $P = 0.0001$). Phyla positively associated with fat but negatively associated with fiber were predominantly Bacteroidetes and Actinobacteria, whereas Firmicutes and Proteobacteria showed the opposite association. However, within each phylum, not all lower-level taxa demonstrated similar correlations with dietary components (fig. S1B). Taxa correlated with BMI also correlated with fat and percent calories from saturated fatty acids (fig. S1B and table S1).

Following the suggestion by Arumugam *et al.* (4) that the human gut microbiome can be partitioned into enterotypes, we investigated whether the 98 COMBO samples partitioned into clusters that were detectably associated with dietary or demographic data (Fig. 2). Several methods for data processing and clustering were compared (fig. S2). In one analytical approach (weighted UniFrac, no lane masking; fig. S2), partitioning around medoids (PAM) analysis favored partitioning into three clusters, although with quite low support (silhouette score 0.2) suggesting that clustering could be due to chance. Comparison to the three genera specified by Arumugam *et al.* (4) showed that relatively high levels of the genera *Bacteroides* and *Prevotella* distinguished two of the clusters, whereas the third showed slightly higher levels of *Ruminococcus*. However, most methods showed two clusters, with stronger support (Fig. 2; Jensen-Shannon distance, silhouette score 0.66), in which the *Bacteroides* enterotype was fused with the less well distinguished *Ruminococcus* enterotype. As described below,

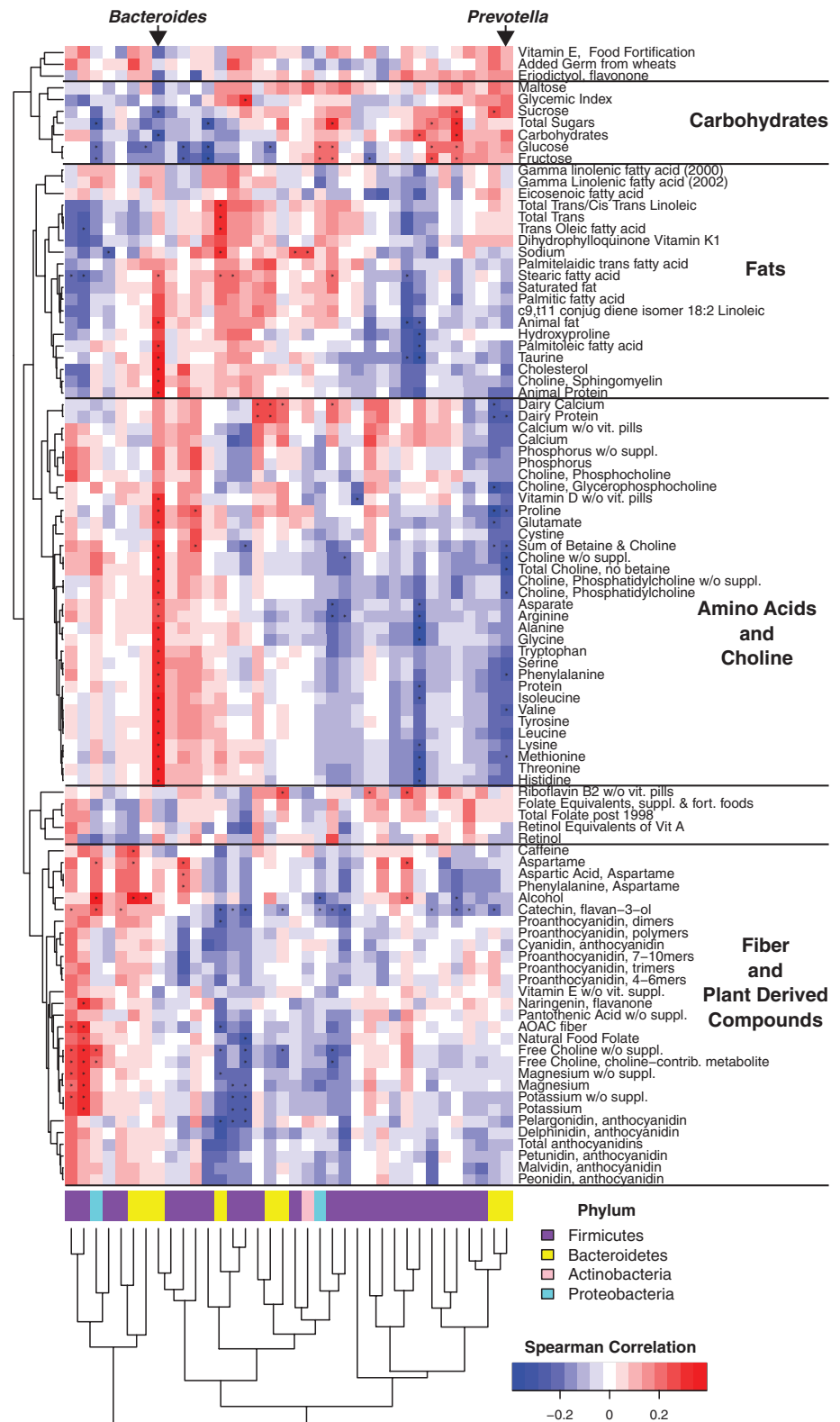


Fig. 1. Correlation of diet and gut microbial taxa identified in the cross-sectional COMBO analysis. Columns correspond to bacterial taxa quantified using 16S rDNA tags; rows correspond to nutrients measured by dietary questionnaire. Red and blue denote positive and negative association, respectively. The intensity of the colors represents the degree of association between the taxa abundances and nutrients as measured by the Spearman's correlations. Bacterial phyla are summarized by the color code on the bottom; lower-level taxonomic assignments specified are in fig. S1. The dots indicate the associations that are significant at an FDR of 25%. The FFQ data were used for this comparison (both FFQ and Recall dietary data are shown together in fig. S1). Columns and rows are clustered by Euclidean distance, with rows separated by the predominant nutrient category.

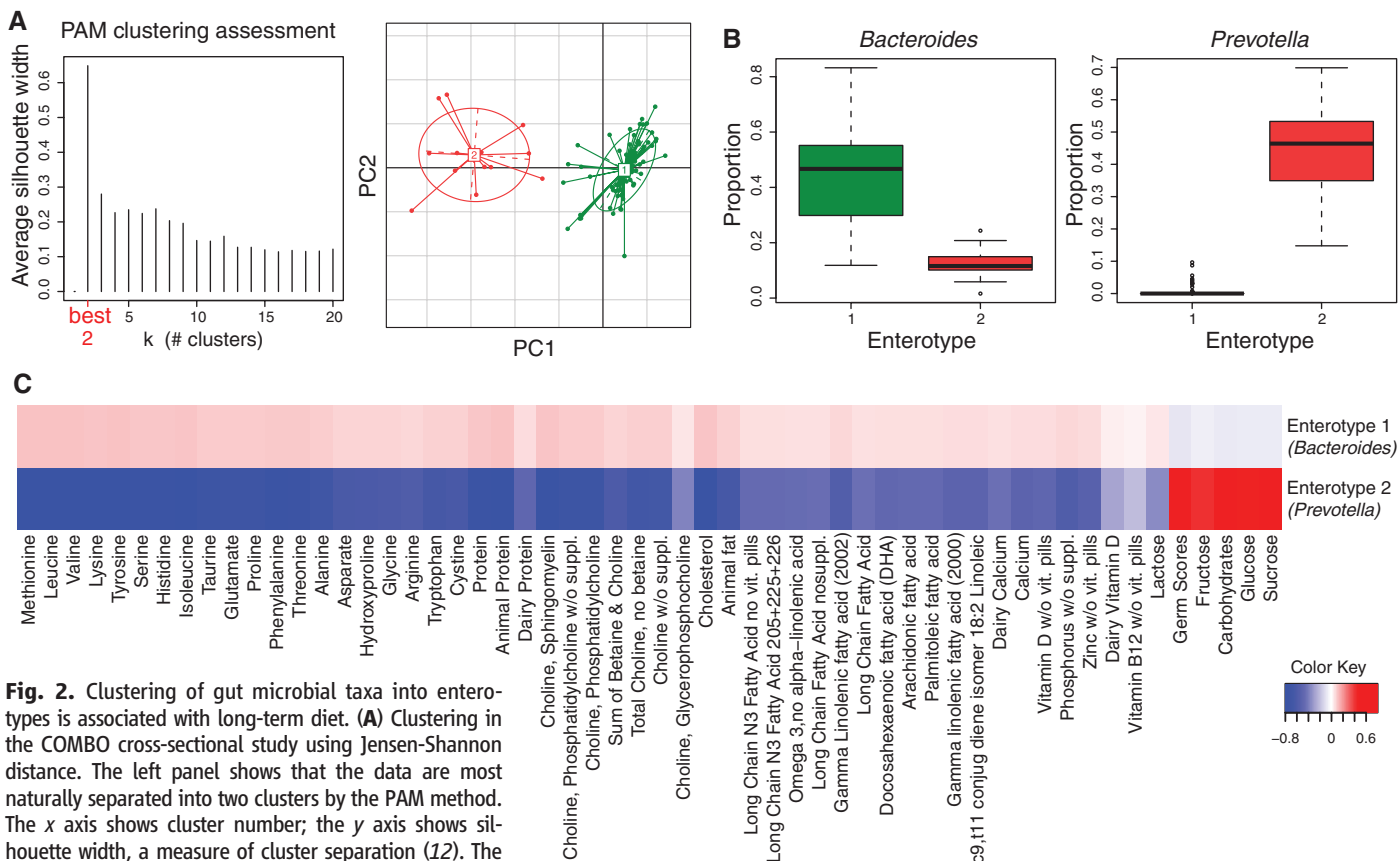


Fig. 2. Clustering of gut microbial taxa into enterotypes is associated with long-term diet. **(A)** Clustering in the COMBO cross-sectional study using Jensen-Shannon distance. The left panel shows that the data are most naturally separated into two clusters by the PAM method. The x axis shows cluster number; the y axis shows silhouette width, a measure of cluster separation (12). The right panel shows the clustering on the first two principal components. **(B)** Proportions of bacterial taxa characteristic of each enterotype. Boxes represent the interquartile range (IQR) and the line inside represents the median. Whiskers denote the lowest and highest values within $1.5 \times$ IQR. **(C)** The association of dietary components with each enterotype. The strength and direction of each association, as measured by

the means of the standardized nutrient measurements, is shown by the color key at the lower right. Enterotype is shown at the right. Red indicates greater amounts, blue lesser amounts of each nutrient in each enterotype (complete lists of nutrients are in table S2). Columns were clustered by Euclidean distance.

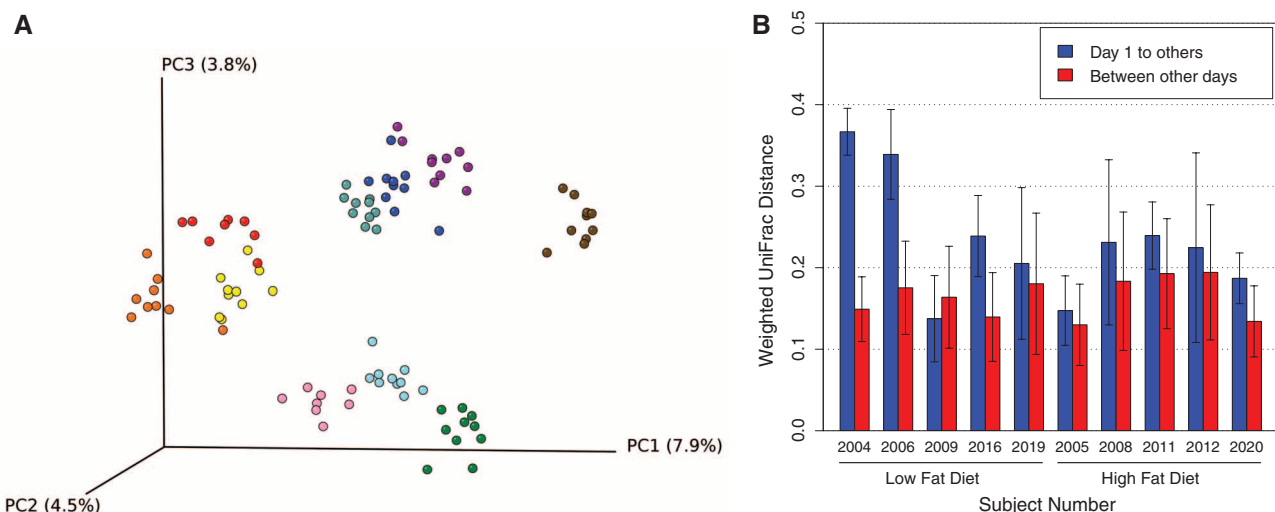


Fig. 3. Changes in bacterial communities during controlled feeding. Ten subjects were randomized to high-fat/low-fiber or low-fat/high-fiber diets, and microbiome composition was monitored longitudinally for 10 days by sequencing 16S rDNA gene tags (CAFE study). **(A)** Cluster diagram-based principal coordinates analysis using unweighted UniFrac. Colors indicate samples from each individual. **(B)** Day 1 samples are outliers compared to all other days,

indicating change in the gut microbiome within 24 hours of initiating controlled feeding. In this analysis, weighted UniFrac distances between samples are compared within subjects in two groups. The first collection of distances compares the day 1 samples to days 2 to 10; the second group compares samples from all days to all others excluding day 1, indicating rapid change ($P = 0.0003$, 10,000 permutations). Error bars indicate 1 SD of the distances.

dietary effects primarily distinguish the *Prevotella* enterotype from the *Bacteroides* enterotype.

At an FDR of 5%, six genera differed between the *Prevotella* and *Bacteroides* enterotypes (fig. S3). The *Bacteroides* enterotype was distinguished by the additional presence of *Alistipes* and *Parabacteroides* (phylum Bacteroidetes). The *Prevotella* enterotype was distinguished by the additional presence of *Paraprevotella* (phylum Bacteroidetes) and *Catenibacterium* (phylum Firmicutes) (fig. S3). The enterotype clustering was driven primarily by the ratio of the two dominant genera, *Prevotella* to *Bacteroides*, which defines a gradient across the two enterotypes (fig. S5).

At an FDR of 25%, nutrients from the long-term FFQ but not the short-term Recall questionnaire were associated with enterotype composition, indicating that long-term diet strongly correlates with enterotype (the relatively high FDR was used to avoid excessively strict filtering and to visualize the full pattern). The *Bacteroides* enterotype was highly associated with animal protein, a variety of amino acids, and saturated fats (Fig. 2C), which suggests that meat consumption as in a Western diet characterized this enterotype. The *Prevotella* enterotype, in contrast, was associated with low values for these groups but high values for carbohydrates and simple sugars, indicating association with a carbohydrate-based diet more typical of agrarian societies. Self-reported vegetarians ($n = 11$) showed enrichment in the *Prevotella* enterotype (27% *Prevotella* enterotype versus 10% *Bacteroides* enterotype; $P = 0.13$). The one self-reported vegan was in the *Prevotella* enterotype. No significant associations were seen with demographic data at this FDR.

A short-term controlled-feeding experiment (CAFE) was carried out to test the stability of the gut microbiome and the observed nutrient-microbiome associations. Ten subjects were sequenced and randomized to high-fat/low-fiber or low-fat/high-fiber diets and were then sampled over 10 days (Fig. 3). Analysis of 16S tag data from stool samples showed that intersubject variation was by far the predominant source of variance in the data (10). Figure 3A shows sharp clustering of the microbiome sequence data by individual in unweighted UniFrac, emphasizing that distinctive lineages are present in each subject. Over 10 days of controlled feeding, there was no reduction in UniFrac distances for stool or biopsy samples between individuals fed the same diet, demonstrating that a short-term identical diet does not overcome intersubject variation.

Remarkably, changes in microbiome composition were detectable within 24 hours of initiating controlled feeding. For each individual sampled, the first sampling day represented an outlier (Fig. 3B; $P = 0.0003$, 10,000 permutations), indicating rapid change. Similar results were seen in the unweighted analysis ($P = 0.0002$). The taxa affected differed among individuals.

The relationship of changes in microbiome composition to the transit time of material through the gut was also investigated. Subjects swallowed x-ray-

opaque markers at the start of the study, allowing quantification of transit time by abdominal x-ray. Transit time was faster with the high-fiber diet (2 to 4 days) than with the high-fat diet (2 to 7 days; $P = 0.02$; two-sided Wilcoxon rank sum test), as expected. All patients retained at least one of the 24 markers 48 hours after the start of the experimental diet. Thus, the changes in microbiome composition, which occurred within 24 hours, were faster than clearance of residual material from the gut.

To probe metabolic functionality during the CAFE study, we also analyzed changes in total gene content using shotgun metagenomics. We compared stool samples from day 1 and day 10 (1.05×10^6 sequence reads total). Sequence reads were annotated for function using the KEGG database (11), then interrogated to assess the taxa and classes of genes present. No significant changes in proportions among archaea, bacteria, and eukaryotes were detected, and bacterial taxa inferred from shotgun metagenomic data paralleled the 16S rDNA data (fig. S4). We investigated gene groups that changed significantly between day 1 and 10 and differed between the high-fat and high-fiber groups. To control for between-subject variability, we used the day 1 samples as within-subject controls, and subtracted each subject's day 1 functional category counts from day 10 samples from that same subject. Functional categories that differentiated diets included bacterial secretion system ($P = 0.01$, t test), protein export ($P = 0.022$), and lipoid acid metabolism ($P = 0.045$), thus indicating bacterial functions potentially involved in responding to these dietary changes.

We next assessed the response of enterotypes to the controlled feeding regimen. Each of the samples from the 10 subjects was assigned to an enterotype category on the basis of their microbiome distances to the medoids (12) of the enterotype clusters as defined in the COMBO data. All subjects started in the *Bacteroides* enterotype (high protein and fat). None switched stably to the *Prevotella* (carbohydrate) enterotype over the duration of the study. A single specimen scored in the *Prevotella* (carbohydrate) enterotype but reverted by the time of the next sample. Thus, over the 10 days of the dietary intervention, we did not see stable switching between the two enterotype groups characterized by the dietary extremes, despite feeding of a low-fat/high-fiber diet to half the subjects.

Finally, several factors were significantly correlated with microbiome composition but not with enterotype partitioning. Examples included BMI, red wine, and aspartame consumption (7). Thus, not all associations between host and microbiota are captured in the enterotype distinctions.

Comparison of long-term and short-term dietary data showed that only the long-term diet was correlated with enterotype clustering in the cross-sectional study. In the interventional study, changes were significant and rapid, but the magnitude of the changes was modest and not sufficient to switch individuals between the enterotype clus-

ters associated with protein/fat and carbohydrates. Thus, our data indicate that long-term diet is particularly strongly associated with enterotype partitioning. The dietary associations seen here parallel a recent study comparing European children, who eat a typical Western diet high in animal protein and fat, to children in Burkina Faso, who eat high-carbohydrate diets low in animal protein (13). The European microbiome was dominated by taxa typical of the *Bacteroides* enterotype, whereas the African microbiome was dominated by the *Prevotella* enterotype, the same pattern seen here. There are, of course, many differences between Europe and Burkina Faso that might influence the gut microbiome, but dietary differences provide an attractive potential explanation. Having confirmed enterotype partitioning and established the association with dietary patterns, it will be important to determine whether individuals with the *Bacteroides* enterotype have a higher incidence of diseases associated with a Western diet, and whether long-term dietary interventions can stably switch individuals to the *Prevotella* enterotype. If an enterotype is ultimately shown to be causally related to disease, then long-term dietary interventions may allow modulation of an individual's enterotype to improve health.

References and Notes

1. L. V. Hooper, J. I. Gordon, *Science* **292**, 1115 (2001).
2. A. Emminger, E. Kahmann, D. S. Savage, *Cancer Lett.* **2**, 273 (1977).
3. S. R. Gill et al., *Science* **312**, 1355 (2006).
4. M. Arumugam et al., *Nature* **473**, 174 (2011).
5. G. D. Wu et al., *BMC Microbiol.* **10**, 206 (2010).
6. M. Margulies et al., *Nature* **437**, 376 (2005).
7. See supporting material on Science Online.
8. C. Lozupone, M. Hamady, R. Knight, *BMC Bioinformatics* **7**, 371 (2006).
9. B. H. McArdle, M. J. Anderson, *Ecology* **82**, 290 (2001).
10. R. E. Ley, P. J. Turnbaugh, S. Klein, J. I. Gordon, *Nature* **444**, 1022 (2006).
11. M. Kanehisa, S. Goto, S. Kawashima, Y. Okuno, M. Hattori, *Nucleic Acids Res.* **32** (database issue), D277 (2004).
12. P. J. Rousseeuw, *J. Comput. Appl. Math.* **20**, 53 (1987).
13. C. De Filippo et al., *Proc. Natl. Acad. Sci. U.S.A.* **107**, 14691 (2010).

Acknowledgments: Supported by NIH grants UH2 DK083981 (F.D.B., J.D.L., and G.D.W.) and R01 AI39368 (G.D.W.); Penn Genome Frontiers Institute; Penn Digestive Disease Center grant P30 DK050306; Joint Penn-CHOP Center for Digestive, Liver, and Pancreatic Medicine grants S10RR024525, UL1RR024134, and K24-DK078228; and the Howard Hughes Medical Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources, National Institutes of Health, or Pennsylvania Department of Health. Accession numbers (Sequence Read Archive): for the CAFE study, SRX021237, SRX021236, SRX020587, SRX020379, and SRX020378 (metagenomic); for the COMBO study, SRX020773, SRX020770, and SRX089367.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1208344/DC1
Materials and Methods
Figs. S1 to S5
Tables S1 to S10
References (14–21)

13 May 2011; accepted 17 August 2011
Published online 1 September 2011;
10.1126/science.1208344