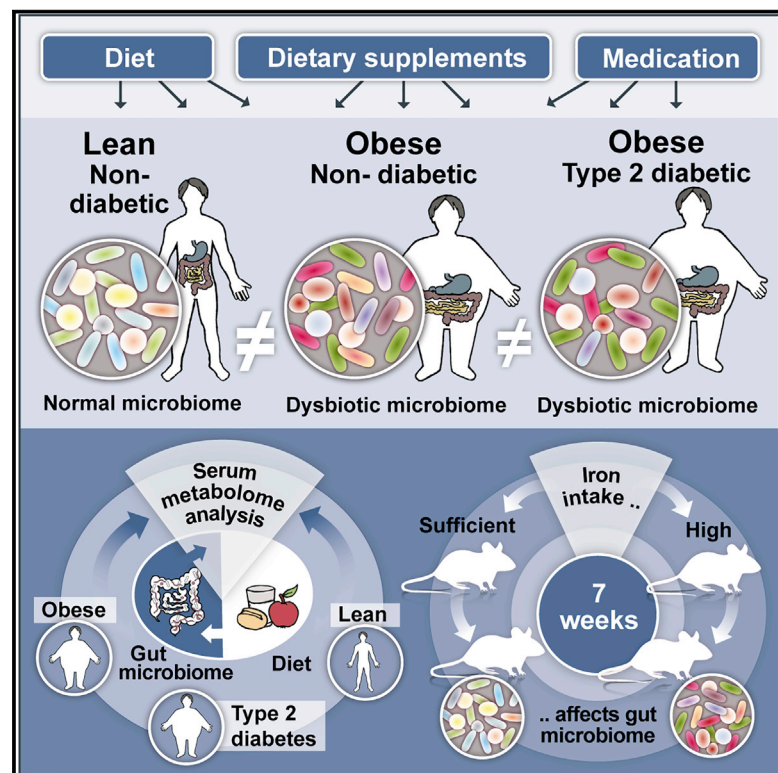# Cell Host & Microbe

Obese Individuals with and without Type 2 Diabetes Show Different Gut Microbial Functional Capacity and Composition

Graphical Abstract

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In Brief

Co-occurrence of obesity and type 2 diabetes poses challenges in assessing microbiota changes specific to each condition. By comparing gut microbiomes of lean, obese non-diabetic, and obese type 2 diabetic individuals, Thingholm et al. found that obesity, unlike type 2 diabetes, was associated with microbiome variation. Medications and dietary supplements were further linked to microbiota alterations.

Highlights

- Obesity, but not type 2 diabetes, associated with gut microbiome variation

- Medications and dietary supplements associated with gut microbiome variation

- High iron intake affected microbiome composition in mice

- Microbiome variation was also reflected in serum metabolite profiles

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Article

Obese Individuals with and without Type 2 Diabetes Show Different Gut Microbial Functional Capacity and Composition

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SUMMARY

Obesity and type 2 diabetes (T2D) are metabolic disorders that are linked to microbiome alterations. However, their co-occurrence poses challenges in disentangling microbial features unique to each condition. We analyzed gut microbiomes of lean non-diabetic (n = 633), obese non-diabetic (n = 494), and obese individuals with T2D (n = 153) from German population and metabolic disease cohorts. Microbial taxonomic and functional profiles were analyzed along with medical histories, serum metabolomics, biometrics, and dietary data. Obesity was associated with alterations in microbiome composition, individual taxa, and functions with notable changes in *Akkermansia*, *Faecalibacterium*, *Oscillibacter,* and *Alistipes*, as well as in serum metabolites that correlated with gut microbial patterns. However, microbiome associations were modest for T2D, with nominal increases in *Escherichia/Shigella*. Medications, including antihypertensives and antidiabetics, along with dietary supplements including iron, were significantly associated with microbiome variation. These results differentiate microbial components of these interrelated metabolic diseases and identify dietary and medication exposures to consider in future studies.

INTRODUCTION

The incidences of both obesity and type 2 diabetes are increasing worldwide, and their comorbidities and medical requirements incur high and rising healthcare costs. Obesity is a risk factor for T2D, but while 86% of individuals with T2D are overweight or obese, not all obese individuals develop T2D (Daousi et al., 2006; Narayan et al., 2007). Multiple factors play a role in the development of these diseases, including genetics, lifestyle, and the gut microbiome, with an increasing body of evidence supporting the microbiome's role in obesity and in T2D (Boulangé et al., 2016; Chobot et al., 2018; Peters et al., 2018; Trøseid et al., 2013; Turnbaugh et al., 2006; Vrieze et al., 2012). However, it remains difficult to disentangle the exact microbial

A   PopGen and FoCus cohort derived microbiome data and covariates

Phenotypic groups
Lean non-diabetic (LH, n=633 16S, n=95 mgx)
Obese non-diabetic (ObH, n=494 16S, n=55 mgx)
Obese type 2 diabetic (ObT2D, n=153 16S, n=51 mgx)

Microbiome data
16S rRNA gene amplicon sequencing data (16S, n = 1280)
Shotgun metagenomic sequencing data (mgx, n = 201)

Metabolomics data
Untargeted measure of serum metabolites (n = 400)

Metadata
Medications (e.g. antihypertensives, antidepressants, antidiabetics)
Nutrition (nutritional variables e.g. protein, fiber, fatty acids)
Food supplements (vitamins and minerals)
Medical histories (current and prior conditions and surgeries)
Biometrics (e.g. age, gender, height, weight)
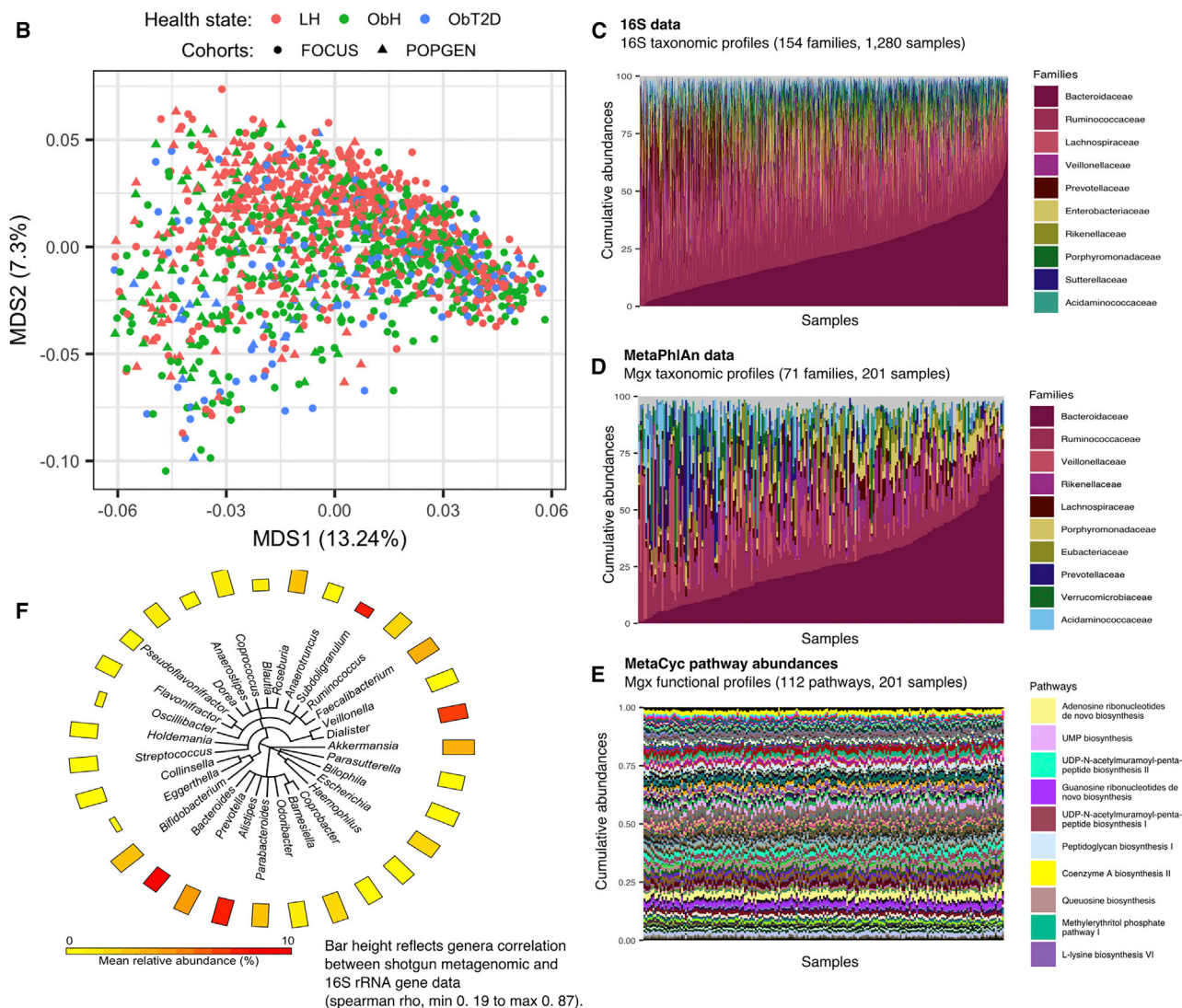
Figure 1. The Gut Microbiome in Obesity and Obesity-Associated type 2 Diabetes in Two Northern German Population Cohorts
We investigated 1,280 individuals from two cohorts (popgen, n = 436, and focus, n = 844) to assess the role of the gut microbiome in T2D and obesity. In addition to extensive lifestyle, dietary, and environmental covariates recorded for these individuals, a stool sample from each participant was assayed using 16S rRNA gene sequencing, and a subset of these samples (n = 201) were metagenomically profiled.
(A) Overview of study data and metadata; detailed information in Table S1.
(B) Ordination of genus-level taxonomic profiles from 16S rRNA gene sequencing, using multidimensional scaling (MDS) of Bray-Curtis dissimilarities.

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features associated with obesity and T2D while also ruling out external confounders. Studies to identify distinguishing features of the microbiome that uniquely characterize each of the two conditions are needed in order to understand whether the progression from obesity to T2D is, in part, mediated by the gut microbiome.

Obesity and diabetes are both metabolic conditions associated with a range of physiological functions closely related to the gut and gut microbiota. Intestinal dysbiosis is a common observation in obesity, while the observation is less consistent in T2D (Aron-Wisnewsky et al., 2019; Le Chatelier et al., 2013; Forslund et al., 2015; Karlsson et al., 2013; Qin et al., 2012; Turnbaugh et al., 2009). Low-grade inflammation and altered levels of lipopolysaccharides (LPS) and short-chain fatty acid (SCFA) have also been associated with metabolic disease (Forslund et al., 2015; Karlsson et al., 2013; Qin et al., 2012). Together, these observations suggest that the development of obesity-associated T2D, characterized by dysregulated glucose metabolism and insulin resistance, could be related to progressive disruption of the gut microbiome after initiation by obesity.

While there is general agreement that ecological diversity and taxonomic composition of the gut microbiome are altered in obesity and T2D, associations with single microbes or microbial products have been inconsistent between studies. These deviations may potentially be due to small sample sizes, differing study designs, geography, and clinical factors (Falony et al., 2016; Finucane et al., 2014; Sze and Schloss, 2016; Yassour et al., 2016). The high inter-individual variability of the gut microbiome, and its sensitivity to environmental influences complicates population-scale microbial research in complex diseases generally, potentially explaining some of these inconsistencies (Falony et al., 2016; Zhernakova et al., 2016). Despite the complications of microbial studies, a number of consistent associations have been observed among them, including an altered abundance of butyrate producing bacteria (Le Chatelier et al., 2013; Forslund et al., 2015; Karlsson et al., 2013; Qin et al., 2012; Vrieze et al., 2012).

In the current study, we performed a detailed analysis of lean non-diabetic ("lean healthy", LH), obese non-diabetic (ObH), and obese T2D (ObT2D) individuals to identify compositional and functional features of the gut microbiome that associate with obesity, as well as those which deviate between obese individuals with and without T2D. We included 16S rRNA gene (16S) sequencing data for 1,280 samples and shotgun metagenomic data for a subset of 201 samples, in addition to extensive covariates describing anthropometrics, nutritional behavior, and medications. All samples in the core analysis are from the northern German cohorts PopGen (Krawczak et al., 2006) and FoCus (Müller et al., 2015), while key findings were replicated in 880 additional individuals from the North-Eastern German SHIP (Study of Health in Pomerania) cohort (Völzke et al., 2011). We identified several associations between the gut microbiome, plasma metabolome, obesity and diabetes phenotypes, and environmental factors. These comprised associations with gut microbial taxa, including a decrease of *Akkermansia*, *Oscillibacter, and Intestinimonas* in obesity, and a nominal increase of *Escherichia/Shigella* specific to T2D, as well as circulating metabolite changes [including branched-chain amino acids (BCAA)]. Dietary supplements, including iron and magnesium, and medications also covaried with microbial composition and functional potential, such as the known strong association with metformin, and we identified further effects of antihypertensive and antiphlogistic medications on the gut microbiome. The effects of dietary iron on microbiome composition were confirmed in a mouse feeding experiment, in which the difference between high and sufficient iron intake recapitulated the diet-associated microbiome divergence observed in human populations.

RESULTS

### Overview of the German Metabolic Disease Cohort

The current study included 1,280 individuals from the Northern German cohorts PopGen (Krawczak et al., 2006) and FoCus (Müller et al., 2015) (Figure 1; Table S1). The PopGen and Focus cohorts have recorded information on medication, diet, and dietary supplement usage, together with an extensive phenotypic characterization including values of age, gender, BMI, and fasting glucose levels (from the PopGen/P2N biobank; see STAR Methods, Table S1). Furthermore, untargeted serum metabolomic profiles were generated for 400 study participants using the Metabolon platform, comprising 390 identified metabolites (STAR Methods). Data and specimens from both cohorts were handled by the same biobank using the same study protocol. Compatibility of the 16S microbiome data between the two cohorts is good as described previously (Wang et al., 2016). All individuals were grouped into three phenotypes: (a) lean (BMI ≤ 25) without diabetes, inflammatory bowel disease (IBD), or irritable bowel syndrome (IBS), with fasting glucose level below 125 mg/dl ("lean healthy", **LH**, n = 633); (b) obese (BMI > 30) with same criteria as LH except for BMI ("obese healthy," **ObH**, n = 494); and (c) obese (BMI > 30) with diagnosed T2D or fasting glucose level above 125 mg/dl, and without IBD and IBS, respectively (**ObT2D**, n = 153; Table S1).

A subset of 201 samples was selected for shotgun metagenomic sequencing. These were targeted to exclude several potential confounders during comparisons of the three populations (LH n = 95, ObH n = 55, and ObT2D n = 51), notably to achieve uniformity of cardiovascular measures (see STAR Methods, Table S1). The ObH group was selected to be generally healthy (despite being obese) as reflected by the uniformity of mean

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(C and D) Family-level taxonomic abundances for 16S (C) and metagenomic (D) data. The top 10 abundant families are annotated in the panel legend, while the remaining detected families are indicated in gray. Samples are ordered according to increasing relative abundance of bacteroidaceae.

(E) MetaCyc pathway abundances across 201 metagenomes (Caspi et al., 2014). The top 10 abundant pathways are colored and annotated in the panel legend, while the remaining core pathways (selected as top 50% mean abundant and top 50% variant) are indicated in gray.

(F) The phyloT-based (http://phylot.biobyte.de/) phylogeny of 31 genera well-detected in both the 16S and metagenomic profiles. The two measurement types generally agreed well (mean spearman ρ = 0.67). A total of 27 core genera from the shotgun data matched best with taxonomically identical taxa in the 16S data. The remaining four genera, *eggerthella*, *blautia*, *oscillibacter,* and *subdoligranulum*, showed highest correlation with a genus from the clostridiales order, predominantly in the unclassified clostridiales. See also Figure S1; Table S1.
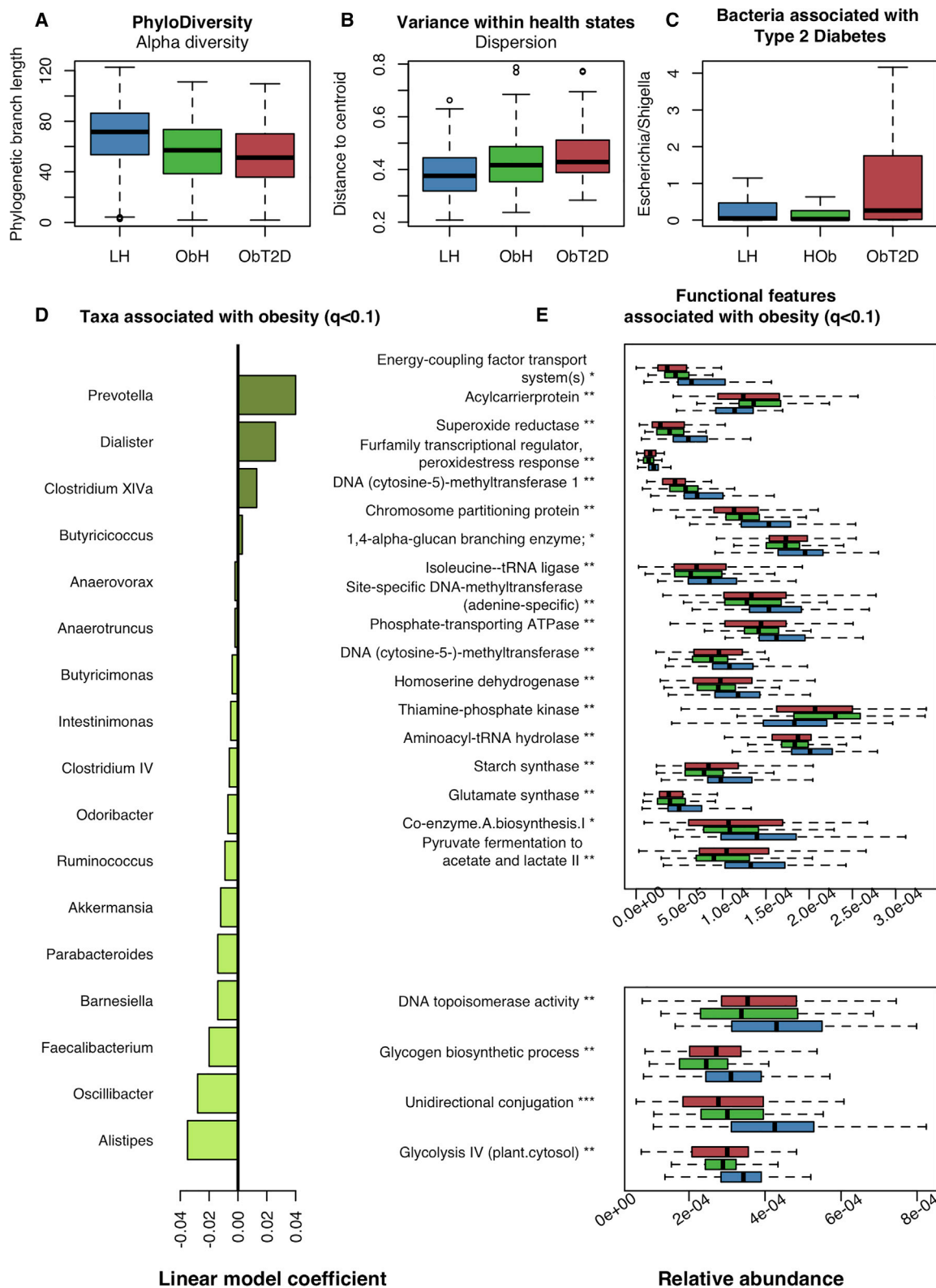
Figure 2. Functional and Microbial Deviations in Obesity and Type 2 Diabetes

(A) Alpha-diversity (total unique phylogenetic branch length) was significantly reduced in obese subjects (compared to LH) (p = 3.20×10⁻¹¹ by robust regression, 16S data) while not significantly different between obese with and without T2D (p = 0.92, ObH versus ObT2D).

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fasting glucose level and blood pressure between the lean and obese non-diabetic subjects. We identified both age and gender as significantly associated with microbiome composition (adonis PERMANOVA, p < 0.001, 16S data, STAR Methods and Supplemental Information), in agreement with previous findings (Falony et al., 2016; Oksanen et al., 2015; Wang et al., 2016; Zhernakova et al., 2016). Accordingly, we corrected for age and gender where possible in the following analyses. In initial comparisons of amplicon- and metagenome-based taxonomic profiles, abundances of genera within subject agreed well across taxa (mean Spearman ρ = 0.67, Figures 1 and S1). Exceptions to this included a small number of taxa (n = 5, of 31 total) with Spearman correlations below 0.5. Four of these five bacteria belonged to the clade Clostridiales, which has been subject to extensive reclassifications, potentially compromising consistent assignments between the two measurement types' taxonomies (Yutin and Galperin, 2013).

In subsequent analyses, six microbiome feature types were studied (additional details in STAR Methods): taxonomic abundances from 16S rRNA gene sequencing [VSEARCH (Rognes et al., 2016)]; species-level taxonomic profiles from metagenomes [MetaPhlAn2 (Truong et al., 2015)]; functional profiles [HUMAnN2 (Franzosa et al., 2018)] summarized as MetaCyc (Caspi et al., 2014) and informative Gene Ontology (iGO) pathways (STAR Methods) and as KEGG Ontology (KO) (Kanehisa et al., 2014) and Enzyme Commission (EC) (Bairoch, 2000) gene families. For each of these six feature types, only the subset of features with the greatest overall mean abundance and standard deviation were analyzed (see STAR Methods), and the dimensionality of functional features (pathways and gene families) was further reduced prior to testing by hierarchical clustering. This resulted in 39 MetaCyc pathway groups, 35 iGO term groups, 38-EC groups, and 78 KO groups used for all subsequent tests (Table S1).

Obesity Is Associated with Specific Gut Microbial Taxa and Functional Capacity, while the T2D Status within Obese Individuals Is Associated with Lower Effect Size

To identify individual microbial features (taxa and functions) associated specifically with obesity and/or with T2D, and not with other covariates (e.g., diet, medications), we assessed each feature's abundances comparing (a) LH with ObH and (b) ObH with ObT2D using generalized linear models in MaAsLin (Multivariate Association with Linear Models; Morgan et al., 2012), with automatic variable selection using boosting as a univariate prescreen (see STAR Methods). For the first analysis (LH versus ObH), covariates were selected from age, gender,

fasting glucose levels, total iron intake (estimated from FFQ and dietary supplements), and medications summarized to the antihypertensive and analgesic classes. For the second analysis (ObH versus ObT2D), covariates were selected from age, gender and BMI, analgesics, antihypertensives, metformin and insulin medications, and the nutrients magnesium and iron intake. These analyses used transformed relative abundance data and implements corrections for sparse, compositional microbial feature data. Sparse (zero-inflated) models were used for taxonomic features with more than 30% zero elements.

One of the simplest summary statistics analyzed as a microbiome feature was alpha-diversity, which was significantly reduced specifically in obesity (p = 3.20×10^{-11} by robust regression, ObH versus LH) and not for ObT2D (p = 0.92, ObH versus ObT2D; Figure 2). This was also the case for composition (beta-diversity) of taxonomic and functional profiles (genera, GO, EC, KO, and MetaCyc pathways; adonis q < 0.1) in obesity, and for taxonomic evaluation of dispersion (genera, betadisper q < 0.1), although not that of functional features (GO, EC, KO, and MetaCyc pathways; betadisper q > 0.1; Figure 2; Table S2). In contrast, when comparing obese subjects with and without T2D, composition was not significantly different across microbial taxonomic profiles or functional features (genera, GO, EC, KO, and MetaCyc pathways; all adonis q > 0.1) after adjusting for diabetic medications. To avoid confounding from metformin and insulin usage, we next evaluated association of compositional variation with fasting glucose levels across all subjects not using metformin or insulin (subsampling from 561 samples). Neither taxonomic profiles nor functional capacity associated significantly with fasting glucose levels (GO, EC, KO, and pathways; adonis q > 0.1). Thus in our cohort, obesity (with normal fasting glucose levels) had a striking association both with taxonomic and functional features of the microbiome, while T2D (in contrast to non-diabetic obesity) had a weak association with microbiome features once diabetic medications were properly considered (Table S2).

When analyzing the association of individual microbial genera (from 16S rRNA gene sequencing) with obesity or T2D, a total of 17 genera were significant with respect specifically to obesity (q < 0.1), including decreased *Akkermansia*, *Faecalibacterium*, *Oscillibacter,* and *Alistipes* (Table S3). The abundant anti-inflammatory species *Faecalibacterium prausnitzii* was decreased in obese individuals (q = 5.29×10^{-3}) while unassociated with T2D (q > 0.1). *Faecalibacterium prausnitzii*, together with *Bacteroides thetaiotaomicron* (itself also obesity-associated), harbor most of the microbial functional features associated with obesity and may therefore be drivers of functional variation (Table S3).

(B) Microbial dispersion was significantly increased in obese subjects as compared to LH (q = 0.023), but not significant between ObH and ObT2D (q = 0.16; Table S2). The illustration was generated using all subjects with 16S data and the betadisper function in R package vegan.
(C) Analysis of individual taxa for association with ObT2D identified limited taxonomic variation in T2D (Table S3). After adjusting for insulin and metformin usage, no genera remained significantly associated after correcting for multiple testing. *Escherichia* possesses properties functionally relevant for T2D, and presented with a nominal increased abundance (p = 0.025) that however was not robust after multiple testing correction.
(D) Analysis of individual taxa for association with obesity identified 17 genera, including *prevotella* and *alistipes* (LH versus ObH, q < 0.1, Table S3). Associations were detected using 16S-based genera abundance profiles and MaAsLin generalized linear model (STAR Methods).
(E) Analysis of microbial processes (MetaCyc, iGO, EC, and KO) identified 22 clusters, comprising 97 features (see Table S3), associated with obesity (q < 0.1). Two plots are used for optimal visualization of all features (top and bottom). One feature from each associated cluster was selected to annotate the panel and may not be the cluster-representative feature. See Table S1 for a full overview of functional clusters. Associated processes were detected using MaAsLin-generalized linear model and metagenomic functional profiles (STAR Methods). Boxplots were made with R function boxplot with default settings (whiskers extend 1.5 times the interquartile range). ***: q < 0.01, **: 0.01 $\leq$ q < 0.05, *: 0.05 $\leq$ q < 0.1. Summary statistics and full lists of associated functional features and taxa are found in Table S3.

Table 1. Association of Commonly Used Medications and Dietary Supplements with Gut Microbiome Composition

A) Medication classes			B) Dietary supplements		
Medication class	p value	q value	Dietary supplement	p value	q value
Genera			Genera		
Antihypertensives	3.85×10^{-4}	9.13×10^{-4} (1.12×10^{-6})	Magnesium	3.54×10^{-4}	5.90×10^{-4}
Analgesics	5.48×10^{-4}	9.13×10^{-4} (3.12×10^{-5})	Multivitamins and mineral	1.15×10^{-4}	3.86×10^{-4}
Antidepressants	6.88×10^{-3}	8.60×10^{-3} (3.33×10^{-2})	Iron	1.56×10^{-4}	3.86×10^{-3}
Antiphlogistics	1.25×10^{-2}	1.25×10^{-2} (8.83×10^{-5})	Vitamin C	7.68×10^{-3}	7.68×10^{-3}
Antidiabetics	6.73×10^{-5}	3.37×10^{-4} (2.75×10^{-5})	Calcium	1.02×10^{-3}	1.28×10^{-3}
Pathways			Pathways		
Antihypertensives	3.31×10^{-7}	8.28×10^{-7}	Magnesium	3.16×10^{-3}	3.07×10^{-3}
Analgesics	4.73×10^{-6}	7.88×10^{-6}	Multivitamins and minerals	1.01×10^{-3}	2.15×10^{-3}
Antidepressants	2.02×10^{-4}	2.53×10^{-4}	Iron	3.85×10^{-2}	3.85×10^{-2}
Antiphlogistics	2.55×10^{-4}	2.55×10^{-4}	Vitamin C	5.32×10^{-4}	2.53×10^{-3}
Antidiabetics	3.21×10^{-11}	1.61×10^{-10}	Calcium	2.69×10^{-2}	3.36×10^{-2}

Linear discriminant analysis (LDA) was performed to evaluate the ability of the gut microbiome to discriminate between users and non-users of (A) five commonly used medication classes and (B) five dietary supplements. P values and q values are given for analyses of samples in the Northern German cohorts (PopGen and FoCus) for medication classes and dietary supplements, for both bacterial abundance (genera) and functional capacity here represented by MetaCyc pathways. In brackets, q values are given for analyses of bacterial abundance in the supporting SHIP cohort (medication only). See STAR Methods for details on the LDA analyses. See also Tables S1 and S4.

Of the 17 associations identified here, 15 were among the analyzed taxa in the independent SHIP cohort, and of these, 7 retained a significant association with obesity (q < 0.1) (Table S3).These obesity-associated taxa include replications of previous studies, thus lending further weight to a small but significant "core" of obesity-associated clades that are common among population contexts (in addition to more variable, population-specific associations). In contrast to obesity, no genera retained a significant robust association with T2D after incorporating the covariates above (i.e., remained differential between ObH and ObT2D individuals, q < 0.1, Figure S2; Table S3). Previous studies have found a positive association between *Escherichia* and both metformin usage and T2D, which we reproduced here in direction (i.e., enrichment) but without statistical significance (Pedersen et al., 2016; Qin et al., 2012; Table S3).

To understand how microbial functional capacities further relate to obesity and T2D, we first tested a group of pathways [from MetaCyc (Caspi et al., 2014) and the informative Gene Ontology, iGO (STAR Methods)] and gene families [as summarized by KO (Kanehisa et al., 2014) and ECs (Bairoch, 2000)] for association specifically with obesity (LH versus ObH). Sixteen gene family clusters and six pathway clusters, together comprising 97 functional features, were associated with obesity (q < 0.1, Table S3). This included a decreased capacity for unidirectional conjugation (Gene Ontology: GO:0009291, q = 3.79×10^{-4}) and superoxide reductase (KEGG Orthology: K05919, q = 1.84×10^{-2}). Conjugation is an important mechanism for bacteria, and the mechanism has been shown to play an important role for the gut microbiome (Sitaraman, 2018). Superoxide reductase catalyzes the conversion of the reactive oxygen species superoxide into the less toxic hydrogen peroxide. A reduction in this capacity indicates a microbiome-induced increase in reactive oxygen species in the intestine of obese subjects. Induction of oxygen stress by microbial dysbiosis has previously been suggested as one of
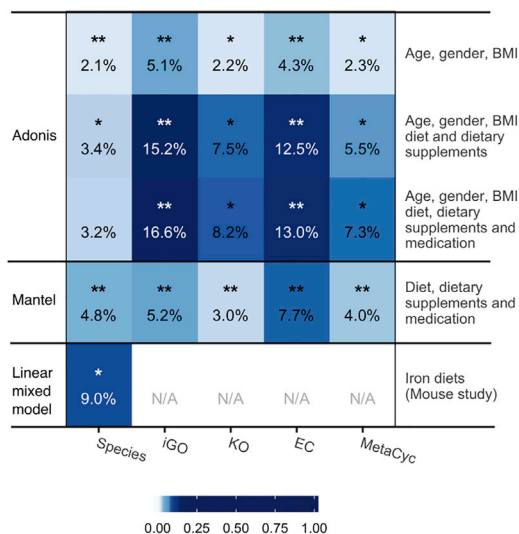
the mechanisms by which the microbiome can cause weight gain and insulin resistance (Qin et al., 2012). Interestingly, while these functionally specific perturbations suggest microbial mechanisms contributing to weight gain, no pathways or gene families significantly differed in abundance between obese subjects with and without T2D after adjusting for multiple testing (q < 0.1, Table S3; Figure S2). This remained true with and without including metformin and insulin in the list of covariates. Thus, we detected little variation in either microbiome functional capacity or in the taxonomic profiles during T2D, after accounting for the separate effects of diabetic medications and of obesity itself.

Commonly Used Medications and Dietary Supplements Associate with Gut Microbiome to the same Extent as Nutritional Variables
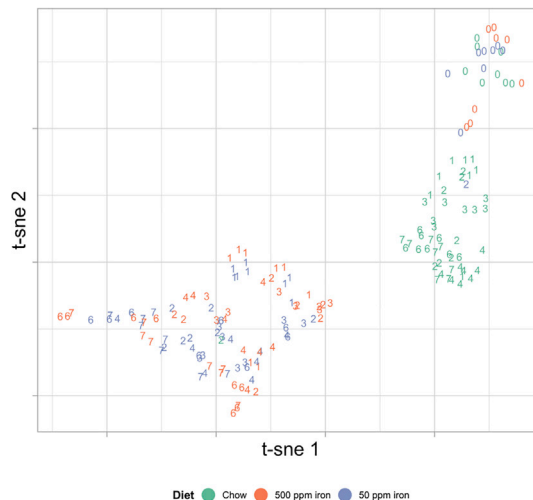
Medication intake is increasingly being linked to microbiome composition, often to a degree as much or more than dietary components, highlighting the importance of considering these exposures in microbiome population studies (Falony et al., 2016; Forslund et al., 2015). We thus evaluated the associations of medication, dietary supplements, and dietary intake with microbiome structure in our cohort (STAR Methods, Figure S4; Table S1). Five main medication classes, comprising analgesics, antidepressants, antihypertensives, antiphlogistics, and antidiabetics, were evaluated and all five were found to be associated with both microbiome structure and functional capacity (summarized as MetaCyc pathways, using linear discriminant analysis (LDA) q < 0.1, see STAR Methods and Table 1). To ensure as equal sample sizes of users and non-users as possible in the analysis, antihypertensives were tested in ObH, while the remaining 4 medication classes were tested in ObT2D subjects (see percent users per group in Table S1). All associations with microbiome structure were supported by the additional independent cross-sectional cohort from North-Eastern Germany

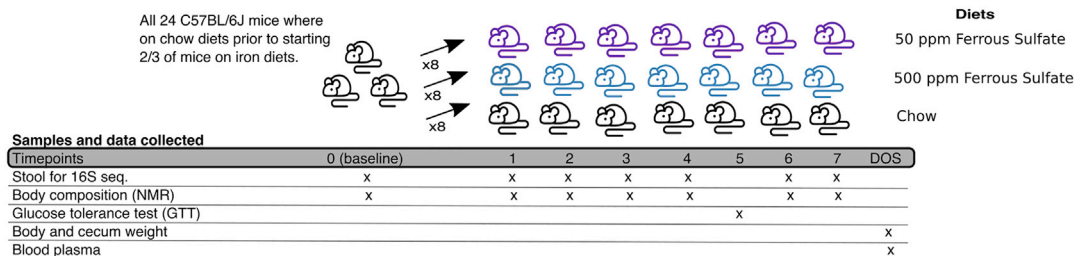**A** Effect of diet on microbiome composition and function

B Overview of mouse study

C Effect of iron diet over time on microbiome composition

Figure 3. Overall Microbiome Composition and Functional Capacity Associate with Diet, Dietary Supplements, and Medication Intake

(A) Evaluation of associations between external factors, comprising dietary nutrients, dietary supplements and medication classes, and the gut microbiome (metagenomic data), showed clear associations albeit with small effects. The percentage of variation explained and the significance of the associations between the gut microbiome (x axis) and external parameters, together with age, gender and bmi (y axis), was evaluated using adonis (top three rows). Furthermore, the correspondence between diet and medication profiles (four drug classes, dietary nutrients, and dietary supplements) and the microbiome, was evaluated using mantel (fourth row). Finally, the effect of high (500 ppm) and sufficient (50 ppm) iron diets on the microbiome composition was evaluated under controlled circumstances in a mouse study using a linear mixed model (fifth row). For adonis, variance explained was calculated using ω instead of r^2 to limit overfitting, and significance was estimated using permutation of samples (see STAR Methods). Percentage variance explained was calculated for mantel analyses as mantel(r)-squared. Both mantel and adonis was performed across all non-diabetic subjects. ***q ≤ 0.001; **0.001 < q ≤ 0.01; *0.01 < q ≤ 0.05.

(B) Schematic overview of the mouse study and collected data. Three groups of eight mice were fed chow for one week (time-point (TP) 0, baseline), after which eight mice were started on an iron diet of 50 ppm ferrous sulfate and another eight mice were started on 500 ppm ferrous sulfate iron. Mice were kept on the respective diets for seven weeks during which stool was collected weekly for microbiome profiling (except week five, week of GTT). Furthermore, extensive phenotypic information was collected on a weekly basis.

(C) Non-linear dimensionality reduction of the mice stool microbiome (Rtsne function in r package Rtsne v0.15 based on OTU tables with Bray-Curtis dissimilarity and perplexity = 10) show clustering of mice on chow and the two iron diets [50 ppm and 500 ppm; (Krijthe, 2015)]. Points are colored by diet and numbered according to time-point (six time-points where mice were kept on different diets and stool collected). The microbiome of mice on different diets are clearly distinct, with the strongest separation between mice on chow versus the iron diets. For one mouse on chow, and one mouse on 50 ppm iron, the second timepoint cluster with the opposite diet group, suggesting that sample IDs for these two samples were swabbed during processing. ppm: parts per million; nmr: nuclear magnetic resonance; DOS: day of sacrifice; iGO: informative gene ontology; KO: kegg ontology; EC: enzyme commission. Figure relates to Figures S3 and S4, Tables 1 and S6.

(SHIP; LDA q < 0.1). For the analysis of dietary supplements, usage (or not) of multivitamins; the minerals magnesium, iron, and calcium; and vitamin C was evaluated in all non-diabetic subjects. The LDA showed that overall gut microbiome taxonomic and functional profiles significantly associated (q < 0.1) with all tested dietary supplements (Table 1). Evaluation of specific taxa and microbial processes (MetaCyc, KO, EC and GO) identified very little specific variation associated with the evaluated dietary supplements and medications, potentially reflecting limited power for such stratified analyses (Table S4).

Evaluation of diet, comprising dietary supplements and nutritional variables obtained from food frequency questionaries (FFQ) data, showed a significant association between any measure of the gut microbiome, taxonomic or functional, and diet across the non-diabetic subjects (PERMANOVA, q < 0.05); however, only a small proportion of variance in the microbiome could be explained by long-term dietary factors, on the order of 1%–10% (Figure 3), in agreement with previous studies (Falony et al., 2016; Wang et al., 2016; Zhernakova et al., 2016). This is also in agreement with our own validation experiments in mice,

in which iron supplementation corresponded with an 8.98% microbiome variation in effect size (see Figure 3; Table S6). Thus under this controlled setting in which mice were fed high or sufficient iron diets over seven weeks, the detectable effect of the change in a single dietary variable was substantially more pronounced. In previous work on the gut microbiome and inflammation, dietary iron intake has been shown to have a unique effect; this is often pro-inflammatory, and increased body iron storage is a risk factor for T2D (Bao et al., 2012; Jaeggi et al., 2015). However, neither total body mass nor glucose tolerance were associated with iron intake in the mice (linear mixed model q > 0.1, Table S6; Figure S3). In the more complex setting of our human population, nutrients, dietary supplements, and medication together corresponded to a similar extent with microbiome taxonomy and function, with limited variation explained by each individual variable (Figure S4). Importantly, these medication classes and dietary supplements are not unique to our cohort; nearly half of adults in similar populations receive at least one prescribed medication per month (CDC, 2017), making any effects on the microbiome of particular interest.

Microbial Associations with Obesity Are Reflected in Serum Metabolite Profiles

The gut microbiome contribute to a range of metabolites that are detectable in serum, providing a direct link between variation in the gut microbiome and the profile of circulating compounds (Liu et al., 2017; Pedersen et al., 2016). Relatedly, a number of serum metabolites have been associated with the development and severity of metabolic diseases (Butte et al., 2015; Libert et al., 2018; Liu et al., 2017). We thus integrated serum metabolite profiles (comprising 390 identified metabolites from a subset of 638 curated peaks, STAR Methods) with microbiome profiles for a subset of the study participants (LH = 228, ObH = 145, and T2D = 27). Overall composition of serum metabolite profiles was significantly associated with that of the gut microbiome in non-diabetic subjects, albeit surprisingly with an extremely low effect size (Mantel test, $r = 0.064$, $r^2 = 0.41\%$, and $p = 0.027$). The limited gut microbial taxonomic variation explained by serum metabolites was less than the variation explained by environmental factors (i.e., diet and medication; $r = 0.18$, $r^2 = 3.43\%$ in the 150 non-diabetic subjects with metagenomic data). These same environmental factors themselves also had a small direct effect on serum metabolites ($r = 0.10$, $r^2 = 1.0\%$, and $p = 0.016$), confirming significant but mainly indirect associations between serum metabolites and both the gut microbiome and dietary and medication factors.

Overall, obesity and T2D were both significantly associated with overall serum metabolome composition (adonis, LH versus ObH $p < 1.00 \times 10^{-4}$, ObH versus ObT2D $p = 1.5 \times 10^{-3}$; Figure 4). Of the 390 identified metabolites, those with <50% prevalence across subjects were considered rare (n = 62) and the remaining abundant (n = 328). A total of 105 individual metabolites (of the 390 tested) were associated with obesity (100 abundant, five rare, q < 0.1; see STAR Methods, Figure 4; Table S5). Among rare metabolites (those with <50% prevalence), three specifically derived from hypertension treatments (oxypurinol, metoprolol, and hydrochlorothiazide) were significantly increased, likely due to comorbidity with obesity (Feig

et al., 2008; Papademetriou et al., 2006). Among the abundant metabolites, glutamate was increased in obese individuals, of interest due to the association between free glutamate and appetite regulation ((Delgado, 2013; Ottosson et al., 2018), Table S5). The concentrations of 19 metabolites (18 abundant, one rare) were significantly different in ObT2D (in contrast to ObH subjects), where all but one were increased (Table S5). Of these metabolites, nine were specific to ObT2D. One metabolite, 3-hydroxyoctanoate, was significantly higher in ObT2D than in ObH, and significantly lower in ObH compared to LH (q < 0.1) (Table S5). 3-hydroxyoctanoate has been assigned anti-lipolytic activity, and found in fasting or diabetic ketoacidosis conditions to reach levels adequate to activate its receptor HCA3, indicating that 3-hydroxyoctanoate plays a role in regulating release of FFA from adipose tissue (Ahmed et al., 2009; Costa et al., 1998; Suzuki and Kaneko-Kawano, 2016). Further associations occurred within BCAA pathways, supporting previous observations associating them with insulin resistance ((Klein and Shearer, 2016; Newgard et al., 2009), Table S5). Intriguingly, the smaller number of specifically T2D-associated compounds corresponds with our findings for the gut microbiome, and may reflect a more restricted or specific molecular alteration overall between obese individuals with and without T2D. However, since our sample size for this comparison is limited to a group of 172 subjects, we acknowledge that power may also be more limited (see Discussion). Instead, we next focused on potential interactions between obesity-associated taxa and small molecules, as both BCAA and glutamate are recognized bacterial metabolites (Ottosson et al., 2018).

We first tested for direct associations of 19 medication-derived serum metabolites with gut microbial composition (as captured by Bray-Curtis dissimilarity). The levels of all three anti-hypertensive derivatives above were significantly associated with the microbiome among their respective users, as well as those of the analgesic metabolite salicylate (adonis q < 0.1, see STAR Methods, Table S5). Next, to assess whether individual microbes were detectably responsible for the associations during obesity (since few taxa were specifically associated with T2D), we directly performed correlation analyses for the 17 taxa and 100 abundant metabolites that were individually differential with respect to obesity (q < 0.1). We first did this, conservatively, only within the LH population using MaAsLin with metabolites as dependent variables (STAR Methods). Nineteen associations passed correction for multiple hypothesis testing (q < 0.1), including ten metabolites from the amino acid superpathway, glutamine, and four lipids. Nine of the metabolites associated with abundance of two taxa in the "anti-inflammatory" Clostridium clusters IV and XIVa (Table S5). Of interest, the association pattern within the ObH individuals differed from the pattern within lean: while the associations in the lean individuals were dominated by Clostridium, this taxon was not found among associations within the obese subjects. Instead, fifteen metabolite-microbe associations were significant within obese subjects, predominantly related to lipid metabolism (n = 8) and amino acids (n = 5) (Table S5). Together, these microbe-metabolite associations in obesity thus suggest a series of specific routes by which taxa and their associated functional roles in the gut might influence systemic signaling, nutrient uptake, and weight gain.
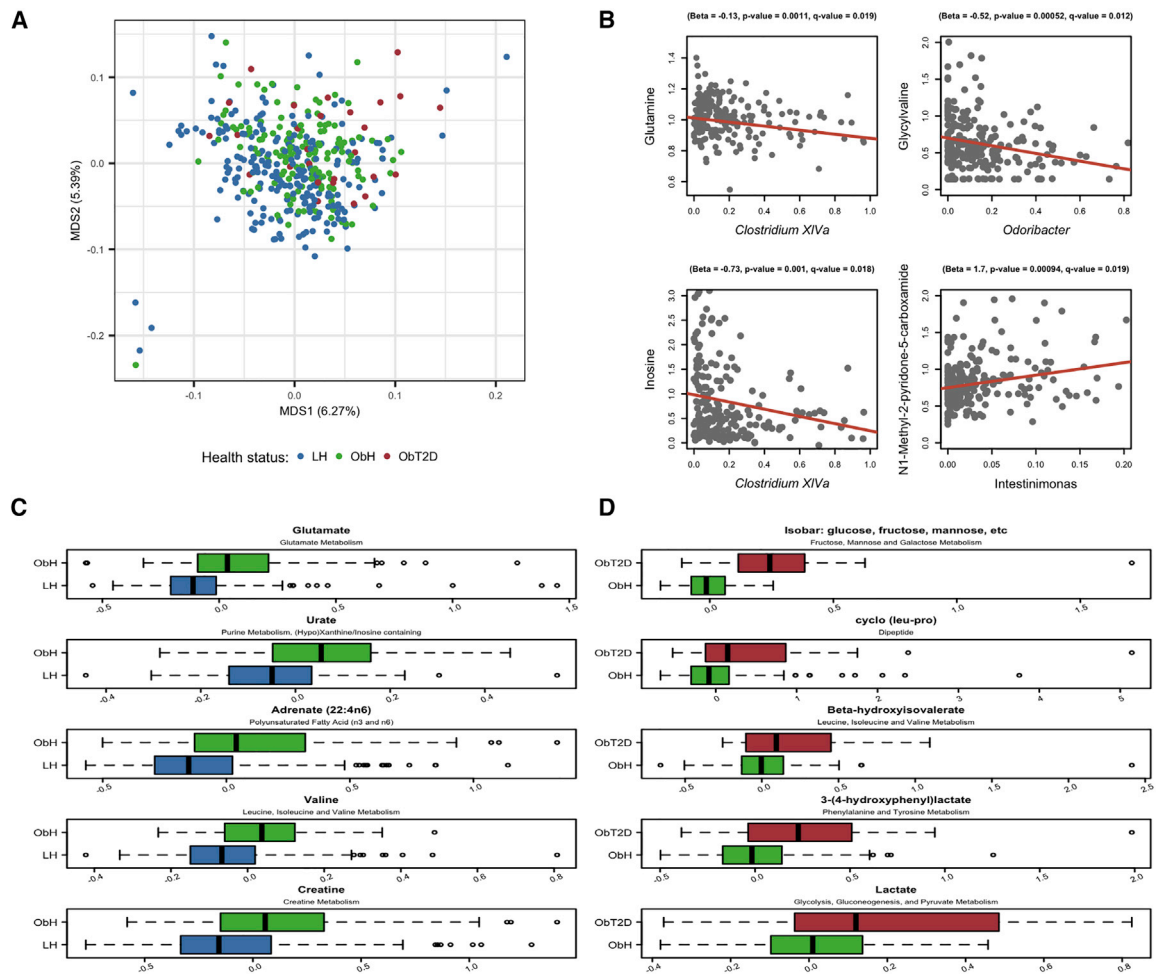
Figure 4. Serum Metabolite Profiles Associated with Obesity and Type 2 Diabetes

(A) Ordination of 400 metabolite profiles across lean non-diabetic (LH), obese non-diabetic (ObH), and obese T2D (ObT2D) individuals, reflecting a shift in the profiles along the first ordination axis, using multidimensional scaling (MDS) based on gower's index (capscale function in r package vegan).

(B) Correlation of four metabolites with single taxon abundance. All genera and metabolites tested for correlations were found in pre-analysis to associate with obesity. The results from MaAsLin analysis provide intercept and slope for the red line and association statistics are given over each plot. Samples are filtered as by maaslin (STAR Methods).

(C and D) A total of 105 metabolites were found to associate with obesity and 19 with T2D (linear model and chi-square test, q < 0.1, Table S5). (C) and (D) show the top five metabolites found by MaAsLin to associate with obesity (LH versus ObH) and T2D (ObH versus ObT2D), respectively. X axes show metabolite residuals after adjusting for age and gender. Boxplots were made with R function boxplot with default settings (whiskers extend 1.5 times the interquartile range). Summary statistics are in Table S5.

DISCUSSION

Ample evidence has shown the gut microbiome to be associated with obesity in humans, and studies in mice suggest that aspects of this association could be causal in its development and persistence. The gut microbiome has also been linked to insulin resistance and physiological parameters related to diabetes, but since these phenotypes are inevitably intertwined with each other and with concomitant medications in humans, the details have been difficult to establish. Here, we analyzed 1,280 gut microbiomes from a defined population in northern Germany to differentiate microbiome links to obesity, obesity-associated T2D, dietary supplements, medication usage, and serum metabolites. This design was uniquely able to determine components of the microbiome associated uniquely with T2D versus non-

diabetic obesity. Comparing obese individuals with and without T2D showed only modest associations between the microbiome and T2D once medication and diet were also factored out, mostly characterized by a nominal increased abundance of *Escherichia/Shigella*. In contrast, comparing lean and obese individuals specifically without diabetes showed compositional differences, as well as differences in functional capacity. Finally, dietary factors, supplements, and medications were individually associated only weakly with components of the microbiome, but in aggregate accounted for a significant amount of variation; they thus have the potential to substantially affect a wide variety of additional microbiome population studies.

Human obesity has proven to be a particularly complex phenotype with respect to the microbiome (Finucane et al., 2014; Le Roy et al., 2018; Sze and Schloss, 2016). This may be

due to the many factors that can influence BMI in human populations, including diet, medication, genetics, and systemic metabolism, which lead to heterogeneity of both clinical outcomes and comorbidities. This multifactorial character of obesity, combined with interpersonal variation in microbial composition and the small effect sizes of single-taxon associations, impede microbiome-obesity studies (Finucane et al., 2014; Sze and Schloss, 2016). A recent meta-analysis, for example, highlighted consistent obesity associations only at the level of whole-community alpha-diversity, and feature-specific classifiers trained on one cohort could not classify obesity in others (Sze and Schloss, 2016). In spite of this, some consistency is emerging, e.g., a decrease in members of Ruminococcaceae and Clostridia in obese subjects, across seven large cohorts including the current study (Peters et al., 2018; Le Roy et al., 2018). The same challenges hold true in associating T2D with the microbiome (Supplemental Information), and any such complex phenotype presents many of the same power, population structure, covariate, and statistical issues as do genetic association studies for complex traits (Falony et al., 2016; Zhernakova et al., 2016). This particularly affected our diabetic obese population, which numbered only 153, in contrast to a greater number of lean or obese non-diabetic subjects. The limited overlap in results among microbiome obesity population studies may both reflect factors such as insufficient power, phenotypic heterogeneity, microbial ecological variability, confounders such as medication, particular susceptibility to geographic or environmental factors (e.g., diet), as well as a broad instability of the disturbed microbiome of obese individuals.

A striking finding from this and several previous studies is the critical role of commonly used medications in modulating gut microbiome structure and function (Falony et al., 2016; Forslund et al., 2015; Imhann et al., 2016; Maier et al., 2018; Zhernakova et al., 2016). While the effects of any single non-antibiotic medication tend to be modest, the aggregate effect of several common medications per individual—and many such medications across a population—can be substantial. Similarly, while single dietary elements appears to be weakly associated with microbial variation (Falony et al., 2016; Zhernakova et al., 2016), in agreement with our study (adonis, 0.1%–0.3%, Figure S4), the combined association of overall diet with vitamin and mineral supplements with microbial variation becomes sizeable. In this space, the notable effect of dietary iron on the microbiome, and its known link with diabetes, prompted us to evaluate its effect in a controlled setting, specifically supplementary feeding in mice (Bao et al., 2012; Lee et al., 2017; Zhao et al., 2017). While direct comparison of affected taxa is not possible between specific-pathogen-free mice and humans, the results confirmed a causal effect of iron intake on the gut microbiome (effect size from linear mixed model, comparing 50 ppm to 500 ppm iron diets, 8.98%), providing support for the observed effect of long-term dietary iron in the human cohort. Thus, larger population studies enable the detection of significant, consistent associations, albeit with small effect sizes for individual compounds, as shown in the current study.

Obesity and T2D are alarming public health issues due to their rapid and continuing increase worldwide, and their etiologies are complex independently of the (also complex) gut microbiome. If host-microbiome interactions are to be targeted as an additional route of understanding these metabolic diseases, the scientific community must continue to study them in greater detail in larger, well-phenotyped cohorts and through additionally well-designed mechanistic studies. This will help to disambiguate the overlapping—but sometimes distinct—microbial effects of the two conditions, in addition to characterization of inter-individual variation in the microbiome as it relates to diet, medications, and other environmental exposures. Key microbial changes may ultimately prove to be context-specific, with distinct functional consequences depending on the host environment, genetics, and stage of temporal progression from health through obesity to T2D.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

A.F., C.H., and L.B.T. conceived the project. U.N., W.L., and M.L. organized recruitment and sample collection for the PopGen and FoCus cohorts. B.F. performed the mouse iron-feeding study and collected samples, guided by AJL and CDV. F.F., M.M.L., G.H., C.O.S., L.B.T., and T.K. organized recruitment and sample collection for the SHIP cohorts or processed and analyzed the SHIP data. Nutritional data were generated and processed by M.K., U.N., and L.B.T. Microbiome data were generated and processed by C.B., M.C.R., and L.B.T.

Medication data were generated and processed by R.B., G.L., L.B.T., and F.F. Statistical analyses were performed by L.B.T., M.H., E.A.F., A.R., M.S., C.H., T.H.K., and J.L.P. provided statistical and methodological advice. L.B.T., M.C.R., C.H., and A.F. interpreted the results. L.B.T. and C.H. wrote the manuscript, with input from all other authors.

DECLARATION OF INTERESTS

SUPPORTING CITATIONS

The following references appear in the Supplemental Information: Lazo de la Vega-Monroy et al., 2013; Singer and Geohas, 2006.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
AlphaTrak2 Blood Glucose Test Strip with an AlphaTrak Glucometer	Abbott laboratories	www.abbott.com
Bruker Minispec (MRI Mouse Body Composition Measurements)	Bruker	www.bruker.com
Eco Medical Systems (MRI Mouse Body Composition Measurements)	Eco Medical Systems	http://www.echomri.com/
ELISA Insulin Kit (Mouse Plasma Insulin)	Alpco	Alpco catalog #80-INSMSU-E10
Enzymatic Assay (Mouse Plasma Glucose)	Stanbio	catalog # 1070-125
QIAamp DNA Stool Mini Kit	QIAGEN	cat. no. 51504
MiSeq Reagent Kits v3	Illumina	MS-102-3003
Nextera DNA Library Preparation Kit	Illumina	FC-141-1007
Deposited Data		
Kiel Cohort Data (Metagenomic Shotgun Sequencing)	This paper	Available upon application from the PopGen biobank (http://www.uksh.de/p2n/Information+for+Researchers.html)
Kiel Cohort Data (16S RRNA Gene Sequencing, Dietary and Medication Data, Phenotypic Characterization)	Wang et al., 2016	Available upon application from the PopGen biobank (http://www.uksh.de/p2n/Information+for+Researchers.html)
Kiel Cohort Data (Medication Data)	This paper	
Kiel Cohort Data (Serum Metabolite Concentrations)	Koch et al., 2017	Available upon application from the PopGen biobank (http://www.uksh.de/p2n/Information+for+Researchers.html)
Mouse Study (16S RRNA Gene Sequencing, Dietary Data and Phenotypic Characterization)	This paper	SRA NCBI, ID: PRJNA550303: Mouse stool bacterial microbiome Raw sequence reads; URL: https://submit.ncbi.nlm.nih.gov
SHIP Cohort Data	Völzke et al., 2011	Available upon application from the SHIP data management unit (https://www.fvcm.med.uni-greifswald.de/dd_service/data_use_intro.php)
Experimental Models: Organisms/Strains		
C57BL/6J Mice	The Jackson Laboratory (Bar Harbor, ME)	000664-C57BL/6J; https://www.jax.org/strain/000664
Software and Algorithms		
Vegan (v2.5-5)	Oksanen et al., 2015	RRID:SCR_011950
Review Environment (v3.5)	R Core Team, 2018	https://www.rproject.org
Sva (v3.30.0)	Leek et al., 2012	https://bioconductor.org/packages/release/bioc/html/sva.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RStudio (v1.1)	R Studio Team	https://www.rstudio.com/
Graphics (v3.5)	Murrell, 2018	https://www.rdocumentation.org/packages/graphics
Lattice (v0.20)	Sarkar, 2008	http://lmdvr.r-forge.r-project.org/figures/figures.html
RColorBrewer (v1.1)	Neuwirth, 2014	https://CRAN.R-project.org/package=RColorBrewer
Caret (v6.0)	http://topepo.github.io/caret/index.html	https://CRAN.R-project.org/package=caret
Stringr (v1.3)	Wickham, 2015	https://CRAN.R-project.org/package=stringr
Plyr (v1.8)	Wickham, 2011	https://CRAN.R-project.org/package=plyr
Dplyr (v0,7)	Wickham et al., 2019	https://CRAN.R-project.org/package=dplyr
Ggrepel (v0.8)	Slowikowski, 2018	https://CRAN.R-project.org/package=ggrepel
data.table (v1.11)	Dowle and Srinivasan, 2019	https://CRAN.R-project.org/package=data.table
Mass (v7.3)	Kafadar et al., 2002	https://CRAN.R-project.org/package=MASS
Metafor (v.2.1)	Viechtbauer, 2010	https://CRAN.R-project.org/package=metafor
reshape2 (v1.4)	Wickham, 2007	https://CRAN.R-project.org/package=reshape2
gridGraphics (v0.3)	Murrell, 2015	https://CRAN.R-project.org/package=gridGraphics
gridBase (v0.4)	Murrell, 2014	https://CRAN.R-project.org/package=gridBase
gridExtra (v2.3)	Baptiste and Antonov, 2017	https://CRAN.R-project.org/package=gridExtra
Grid (v3.5)	R Core Team, 2018	https://stat.ethz.ch/R-manual/R-devel/library/grid/html/grid-package.html
FastTree (v2.1)	Price et al., 2010	http://www.microbesonline.org/fasttree/
Rtsne (v0.15)	Krijthe, 2015	https://github.com/jkrijthe/Rtsne
Mothur	Schloss et al., 2009	https://www.mothur.org/
Robust (v0.4)	Wang et al., 2017	https://CRAN.R-project.org/package=robust
ade4 (v1.7)	Chessel et al., 2004	https://CRAN.R-project.org/package=ade4
lme4 (v1.1.19)	Bates et al., 2015	https://CRAN.R-project.org/package=lme4
lmerTest (v3.0)	Kuznetsova et al., 2017	https://CRAN.R-project.org/package=lmerTest
MuMln (v1.42)	Bartoń, 2018	https://CRAN.R-project.org/package=MuMln
HUMAnN2 (v0.11.1)	Franzosa et al., 2018	https://bitbucket.org/biobakery/biobakery/wiki/humann2
PhyloT	http://phylot.biobyte.de	http://phylot.biobyte.de
MetaPhlAn2	Segata et al., 2012	https://bitbucket.org/biobakery/biobakery/wiki/metaphlan2
MaAsLin	Morgan et al., 2012	https://bitbucket.org/biobakery/biobakery/wiki/maaslin
DeconSeq	Schmieder and Edwards, 2011	http://deconseq.sourceforge.net/
Trimmomatic	Bolger et al., 2014	https://github.com/timflutre/trimmomatic
RDP Classifier	Wang et al., 2007	https://rdp.cme.msu.edu/
Uparse	Edgar, 2013	https://drive5.com/uparse/
UTAX	Robert C. Edgar, Independent Investigator	https://www.drive5.com/usearch/manual8.1/utax_algo.html
SINTAX	Edgar, 2016	https://www.drive5.com/usearch/manual/cmd_sintax.html
VSEARCH	Rognes et al., 2016	https://github.com/torognes/vsearch
Sickle	Joshi and Fass, 2011	https://github.com/najoshi/sickle

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Chow Diet	LabDiet PicoLab Rodent Diet 20, St. Louis, MO	Cat #5R53*
50 ppm Ferrous Sulfate Diet AIN93M Defined Diet with Microcrystalline Cellulose as the Fiber Source.	Dyets, Bethlehem, PA	Cat #115838
500 ppm Ferrous Sulfate Diet AIN93M Defined Diet with Microcrystalline Cellulose as the Fiber Source	Dyets	Cat #115839

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andre Franke (a.franke@mucosa.de). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study and Data Overview

The current study considered 1,280 individuals from the northern German cohorts PopGen (n=436; (Krawczak et al., 2006)) and FoCus (n=844; (Müller et al., 2015)). 16S rRNA amplicon sequencing data were generated for the total population, while 201 individuals were selected for shotgun metagenomic sequencing. The study further included data on medication, dietary intake and supplement usage, nutrition and a phenotypic characterization (Table S1). For analysis, samples were divided into three phenotypic groups: a) lean (BMI $\leq$ 25) without diabetes, ("lean healthy," **LH**); b) obese (BMI >30) without diabetes ("obese healthy," **ObH**); and c) obese (BMI >30) with T2D (**ObT2D**). To asses overall microbiome differences between the phenotypic groups, as well as association with fasting glucose levels and the role of antidiabetic medication, the PERMANOVA-like approach available through the adonis function and the betadisper function for evaluation of difference in dispersion between groups was used, both available in the R package vegan. To identify individual microbial features (taxa and processes) associated specifically with obesity and/or with obesity-associated T2D phenotypes, we assessed each feature's abundance across (a) LH versus ObH and (b) ObH versus ObT2D using generalized linear models in MaAsLin (Multivariate Association with Linear Models) while controlling for clinical covariates (e.g. medication and age) (Morgan et al., 2012).

Further analyses were performed to assess the association of medication, nutrition and dietary supplements with features of the gut microbiome. This included evaluating if gut microbiome taxonomic and functional profiles could discriminate between users and non-users of commonly used medications and dietary supplements, including iron, magnesium, antihypertensives and antidiabetics. The evaluation was performed using LDA, and supported identification of covariates for the analysis of obesity and T2D. To further evaluate the associations between iron intake and the gut microbiome that we observed in the human cohorts, we performed a diet intervention study in 24 C57BL/6J mice. All 24 mice were fed a chow diet for 4 weeks and then separated into groups of 8 as they started on study diets; one group continued on the chow diet, another 8 mice were started on a 50 ppm ferrous sulfate diet, and the remaining 8 mice were started on a 500 ppm ferrous sulfate diet. The mice were kept on these diets for an additional seven weeks. Mouse body composition measurements and stool were collected just prior to starting the mice on the study diets and then weekly thereafter. The collected stool was subjected to 16S sequencing to further evaluate the associations between iron intake and the gut microbiome.

As the effect of a dysbiotic gut microbiome on the host may in part be reflected in serum metabolite profiles, and as many serum metabolites has previously been related to obesity and T2D, we further profiled the serum metabolome and related the profiles to health states and the associated microbial variations. Finally, 16S rRNA amplicon sequencing data for 880 individuals in a second independent cohort, SHIP, was included to evaluate the robustness of associations where data availability allowed. As combining different cohorts can be a source of confounding, all analysis of the SHIP cohort has been kept separate from the analysis of the main study population selected from the PopGen and Focus cohorts. In particular, no analyses rely on data that simultaneously spans PopGen/FoCus and SHIP; instead, primary analyses are carried out only in PopGen/FoCus, with validation tests run separately within SHIP. SHIP is an independent cohort recruited in the area of Greifswald (North-Eastern Germany), using the same protocol for sequencing however with deviations in preprocessing of samples as for PopGen/FoCus (Frost et al., 2019).

Kiel FoCus and PopGen Cohorts

Study samples were selected from the second examination cycle of the PopGen cohort (n=436) (Krawczak et al., 2006) and the cross-sectional and clinical subset of the FoCus cohort (n= 844) (Müller et al., 2015). Samples were collected as described in Wang et al. (Wang et al., 2016). Information on subjects age, sex, BMI etc. can be found in Table S1.

Samples were selected for 16S rRNA amplicon sequencing as follows. Samples with missing data for age, gender and BMI were excluded. From among the remaining samples, non-diabetic lean samples were selected by requiring individual BMI ≤ 25, no diagnosis of IBD or IBS, fasting glucose level ≤125 mg/dl and no self-reported physician diagnosis of diabetes. Non-diabetic obese individuals were filtered by the same filtering criteria except a BMI >30. Finally, obese individuals with T2D had no IBD or IBS diagnosis, BMI >30 and were diagnosed with T2D or has fasting glucose levels above >125 mg/dl. Medication data were curated, manually checked for spelling and consistency, and grouped into analgesics, antihypertensives, antidepressants, antibiotics, antiphlogistics, and antidiabetics (details provided in Supplemental Information).

A subset of samples from each group was selected for shotgun metagenomic sequencing as follows. To minimize potential confounding effects, individuals reporting chronic diarrhea, use of hormone replacement therapy, coronary heart disease, cardiac or venous insufficiency, or thrombotic embolism were excluded. Additional individuals were excluded if they reported a current illness, with the exception of a common cold lacking antibiotics treatment. For individuals where data on CRP was available, non-diabetic samples were selected for normal CRP levels (<5 mg/l). The resulting LH and ObH health groups had compatible fasting glucose levels, and all groups had compatible diastolic and systolic blood pressure levels with slightly lower systolic blood pressure in the LH group (Table S1).

Written, informed consent was obtained from all study participants, and all experiments were approved by the institutional ethical review committee in adherence with the Declaration of Helsinki Principles.

SHIP

Participants of SHIP (the Study of Health in Pomerania) (Völzke et al., 2011) were selected from among the 1,904 individuals with available fecal 16S rRNA amplicon sequencing data from both the SHIP-TREND and SHIP2 arm of the SHIP cohort. A subset of these samples that included medication data and the phenotypic variables age, gender, weight and height was selected for further study. Samples were grouped as obese or lean using the BMI-based definition introduced above. Samples were grouped as diabetic or not diabetic based on information from two variables in SHIP-TREND (T0) ('nn_diab_01' which informs whether diabetes has been diagnosed, and 'diabetes_typ2_t0' which informs whether T2D can be diagnosed based on the data) and one variable for SHIP (S2) ('nn_diab_01'). We note that the variable 'nn_diab_01' does not distinguish between type 1 and T2D, however with the additional requirement of a BMI > 30 we anticipate the majority of individuals will have T2D and not type 1 diabetes. Three samples were removed due to antibiotic usage. These criteria resulted in the selection of 880 samples: 399 lean non-diabetic, 408 obese non-diabetic and 73 obese diabetic. All participants provided written informed consent and the study was approved by the ethics committee of the University Medicine Greifswald.

Mouse Iron-Feeding Study

All animal experiments were approved by the UCLA Animal Care and Use Committee, in accordance with PHS guidelines. The protocol number is ARC #1992–169 (Approval Period from 6/6/2016 through 11/25/2018). Twenty-four male C57BL/6J mice were obtained by the Lusis lab at UCLA from The Jackson Laboratory (Bar Harbor, ME) at 3 weeks of age. The mice were housed four per cage and fed a chow diet (LabDiet PicoLab Rodent Diet 20, cat #5R53*, St. Louis, MO) for one week. During this time, the bedding and feces were mixed together at the end of each day and redistributed among all the cages, in order to minimize variation in baseline intestinal microbial composition.

At 4 weeks of age, 2 cages of mice (8 mice in total) were continued on the chow diet, while 2 cages (8 mice total) were started on a 50 ppm ferrous sulfate diet, and 2 cages (8 mice total) were started on a 500 ppm ferrous sulfate diet. These latter two diets were AIN93M defined diets with microcrystalline cellulose as the fiber source (Dyets, cat #115838 and cat #115839, Bethlehem, PA). Mice were kept on these diets for an additional seven weeks. Mouse body composition measurements and stool were collected just prior to starting the mice on the study diets and then weekly thereafter. Body composition (total fat, muscle, and free fluid) was measured by MRI in a Bruker Minispec with software from Echo Medical Systems, Houston, TX. Total body weight (g), and percentage of fat, muscle and free fluid were calculated. For the stool collection, each mouse was placed in an individual cleaned cage with no bedding for ~5-10 minutes. Feces dropped in each cage were collected for each mouse and snap frozen. Stool and body composition measures were again collected the day before sacrifice (DBS).

After four weeks on the study diets, a glucose tolerance test (GTT) was performed. For the GTT, mice were fasted in clean cages with free access to water for six hours from 7am-1pm. The tip of a mouse's tail was then nicked with a razor blade and the second blood drop was analyzed using an AlphaTrak2 blood glucose test strip with an AlphaTrak glucometer (Abbott laboratories). The mouse was then dosed IP with 1g glucose per kg mouse body weight with a sterile 10% solution of Beta-D(+) glucose in phosphate buffered saline (PBS). At 15, 30, 60, and 120 minutes after dosing, an additional blood sample from the nick was analyzed as described above. The GTT was performed on 3 mice per group on day 1, 3 mice per group on day 2, and 2 mice per group on day 3 after 4 weeks on the study diet). The week of the GTT, neither stool nor body composition measures were collected.

At the day of sacrifice (DOS), mice were anesthetized by inhaled isoflurane, and then body weight was measured. Blood was collected from the retroorbital plexus into lithium heparin tubes (BD Microtainer, catalog # 365971) to measure plasma insulin and glucose levels. Blood samples were centrifuged, and plasma was removed and snap frozen. The cecum was excised and weighed. Plasma insulin was measured using an ELISA insulin kit (Alpco catalog #80-INSMSU-E10). Plasma glucose was measured by an enzymatic assay (Stanbio, catalog # 1070-125).

METHOD DETAILS

Medication Variables for SHIP

Medication variables were defined based on ATC codes. The ATC codes used were: 'Analgesic' (N02 and M01A); 'Antihypertensives' (C02, C09 and C07); 'Antidepressant' (N06A); 'Antibiotics' (A07AA, G01AA, G01BA, J02AA, J04AB, L01DC, R02AB and S01AA); 'Antiphlogistics' (A07E, S01B, V10A and L04A); 'Antidiabetics' (A10).

Medication Variables for PopGen and FoCus

Information on medication usage was obtained for each study subject as free text. To convert this information into a data format that could be implemented in the statistical analyses, a classification tool was constructed and used to classify medication for samples with 16S rRNA gene sequencing data. The classification tool consists of scripts written in PERL and Visual Basic for Applications (VBA) in MS Excel.

Curation of free text and annotation of drug names: In the first step, PERL scripts were used to extract and to annotate relevant data from the free text on medication usage. Stop words, abbreviations and other irrelevant words (e.g. names of pharmaceutical companies) were filtered out by several process steps. The remaining text was parsed through the German ABDA database to find drug names. Each hit was annotated by further information from ABDA and KEGG DRUGS, and resulting summaries saved in Excel sheets divided in three quality categories. The three categories were defined as a) "exact matches" given when the complete drug name was found in the free text, b) "matches" given when the main, relevant part of the drug name was found and c) "approximate matches" found by similarity search in order to correct words caused by typos in the origin text.

Assignment of drug classes: In the next step, a VBA script was used to further classify the drug names according to a set of rules and the obtained annotations. The rules were generated in two steps. First, information from the manually classified medication data was used (medication data for samples with shotgun metagenomic data was manually classified), then the list of rules was extended by 'training the tool' - rules were added for unclassified drugs after each run. The rules were based on the annotations obtained from the searched databases and were designed as follows: An output field from a database was specified together with a search term e.g. "Look for the term 'Analgesic' in the field 'Activity' in the database 'KEGG DRUGS'". If a match was found, a result term was written to the field 'High Level' (HL). A similar rule, extended to include the content assigned to HL, assigned a term to the field 'Low Level' (LL). HL rules would assign a higher drug class such as 'Antihypertensives' or 'Hormones', while the LL rules would assign a subclass such as 'Diuretics' or 'Thyroid hormones'.

Manual evaluation of classification tool: Each drug was assigned a HL and LL term using each rule resulting in multiple result rows per drug. Identical rows were automatically concatenated into a single row, while drugs assigned deviating results was left for the subsequent manual evaluation. Output from the automated classification tool was manually evaluated by looking through rows for errors and unassigned drugs which were then assigned a HL and LL term by searching the databases manually. Based on this tool all drugs listed in the medication data which belonged to the drug classes of interest were identified, and each sample was assigned '1' for 'using' if one or more of the drugs were reported for that sample, and '0' if no match was found.

16S and Shotgun Metagenomic Sequencing

Fecal samples were subjected to 16S rRNA amplicon sequencing as described in Wang et al. (Wang et al., 2016) for FoCus and PopGen. In summary, bacterial genomic DNA was extracted using the QIAamp DNA Stool Mini Kit from QIAGEN and the V1-V2 region of 16S rRNA gene was sequenced on the MiSeq platform with MiSeq Reagent Kits V3. Stringent demultiplexing was carried out by allowing no mismatches in either index sequence. For a subset of 201 individuals, the same DNA extracts were subjected to shotgun metagenomic sequencing as described in Wang et al. (Wang et al., 2016). Briefly, these samples were prepared following the Illumina Nextera DNA Library Preparation Kit and sequenced on the HiSeq Platform as 2×125 bp paired-end reads. Preprocessing of SHIP samples was performed as described in (Frost et al., 2019) and with the same protocol for 16S rRNA amplicon sequencing as for the FoCus/PopGen samples.

16S rRNA Gene Data Processing

Raw 16S rRNA amplicon reads for the Focus and PopGen samples were end-trimmed using sickle in PE mode with a sliding window of 0.1 × read length to maintain average quality ≥ 20. Reads <100 nts after trimming were discarded. Paired reads were then stitched using VSEARCH (Rognes et al., 2016) with minimum length 280 and maximum length 350bp. VSEARCH was further used to filter reads with more than 1 expected error and FastX-Toolkit::fastq_quality_filter was used to filter reads with more than 5% of nucleotides with a quality score below 30. Remaining reads were converted to FASTA format and subjected to chimera filtering using VSEARCH and the gold.fa database. Remaining reads were classified using the UTAX algorithm with RDP database and reads classified as either chloroplast or not classified at domain level were removed.

OTU tables were generated using the UPARSE algorithm (Edgar, 2013) implemented in VSEARCH software and all reads across samples. For reads with multiple exact sequence copies (replicates) only one copy was kept, and read only occurring once across all samples (singletons) were removed, as they were considered likely to be sequencing artefacts. The remaining reads were then clustered at 97% similarity. OTU representative sequences were again checked for chimeras using VSEARCH in de-novo mode. All reads per sample were then mapped to representative sequences using VSEARCH to generate OTU abundance tables. Representative sequences were additionally taxonomically annotated using the RDP database and the RDP classifier (Wang et al., 2007) to the

lowest possible level with minimum 80% bootstrap confidence. OTUs with identical taxonomic annotations were then grouped into taxonomic bins. Samples with fewer than 10,000 reads were removed. Overgrowth of oxygen-tolerant Proteobacteria was quality controlled by excluding samples which fell above the third quartile plus three times interquartile range (IQR) of phylum abundance (n=26).

Raw 16S rRNA amplicon reads for the SHIP cohort were processed as described above except that SINTAX (Edgar, 2016) was used for annotating sequences, and samples were rarefied to 10,000 reads per sample.

Shotgun Metagenomic Processing

Metagenomic sequencing data were quality controlled by requiring a minimum of 4 M paired-end reads per sample after Nextera library adaptor removal (Trimmomatic (Bolger et al., 2014)), trimming of low quality ends (Sickle (Joshi and Fass, 2011)), and removal of host-reads (DeconSeq (Schmieder and Edwards, 2011)). Quality control methods were run with default parameters. Metagenomic samples were taxonomically profiled using MetaPhlAn2 (Segata et al., 2012) and functionally profiled using HUMAnN2 v0.11.1 (Franzosa et al., 2018), both with default settings plus the addition of '–bt2_ps very-sensitive' for the '—metaphlan-options' provided to HUMAnN2. Species from taxonomic profiles were retained for further analysis if their mean relative abundance exceeded 0.005 (0.5%) across the dataset with a minimum abundance of 0.05 (5%) in at least one sample and non-zero abundance in at least 60% of samples.

Tables of pathway and gene family abundance obtained using HUMAnN2 were normalized to relative abundance using 'humann2_renorm_table –units relab' including unmapped and unintegrated (i.e. not assigned to a pathway) read mass. Samples potentially affected by oxygen exposure were removed (identified as described below).

Pathway abundance files were joined into one abundance table and filtered to contain the pathways with top 50% mean abundance and top 50% variance (final n=112). To reduce redundant testing, pathways were clustered using Ward hierarchical clustering (R function hclust in package stats) with dissimilarity 1-Spearman correlation coefficient. Pathway clusters were defined at height 0.6 using the R function cutree in package stats and a representative pathway was selected for each cluster (defined as the pathway with the median mean abundance, Table S1).

Gene family files were regrouped using the humann2_regroup_table command with 'uniref90_level4ec' (Enzyme Commission), 'uniref90_infogo1000' (informative Gene Ontology, iGO) and 'uniref90_ko' (*KEGG Orthology*) terms. iGO is computed specifically for HUMAnN2 as informative subsets of GO build on UniProt's annotations and the structure of the GO hierarchy (Ashburner et al., 2000). Regrouping was performed in order to join gene families with similar function or properties, and thereby reduce both the number of low-abundance variables and the number of tests to be performed downstream. Regrouped, per-sample abundance profiles were joined into three abundance tables and filtered to contain features with top 25% mean abundance and top 25% variance. This filtering returned 453 KO terms, 110 iGO terms and 132 EC terms. For KO, the terms with 'subunit.ribosomal.protein' were removed before filtering in order to further reduce the multiple testing burden. Features from these tables were then clusters (and representative features selected) following the procedures outlined above for metabolic pathways, resulting in 38 EC, 35 iGO and 78 KO clusters (Table S1).

Nutrition Data

Dietary intake during the year preceding examination was assessed in participants of the FoCus and PopGen cohorts via a validated, self-administered food frequency questionnaire (FFQ) (Nöthlings et al., 2007). Nutrient intakes (e.g. dietary protein content) were obtained using the German Food Code and Nutrient Database (vII.3) and provided by the Department of Epidemiology of the German Institute of Human Nutrition Potsdam-Rehbrücke (Dehne et al., 1999). Vitamin and mineral intake from the dietary supplement data was incorporated in downstream analyses in one of two ways: Alone as categorical variable (e.g. in the LDA analyses), and as continuous variables of "total intake" obtained by combining dietary supplement data with the nutritional data derived from FFQ records of intake of food groups (e.g. as covariates in the MaAsLin analyses). As there exists some uncertainty as to the reproducibility of some nutrient groups when assessed with FFQs, and as many nutritional variables correlate, a subset of higher level variables were selected for inclusion in the downstream analyses (Martinez et al., 2013; Nagel et al., 2007). Selected variables with summary statistics are shown in Table S1.

The nutritional variables for mineral and vitamin intake analyzed for association with the microbiome using MaAsLin and adonis included intake originating from diet and supplements. Data on supplement usage was recorded as binary data, which we recoded to amount per day by estimating the typical amount of each compound in commonly used supplementary products in Germany. The supplements evaluated were multivitamin/mineral supplements, and individual supplements including folic acid, iron, zinc, magnesium, calcium, selenium, vitamin B_6, vitamin B_7 (biotin), vitamin B complex (containing all 8 B vitamins), vitamin E, carotene, vitamin A and vitamin C. For each type of supplement, we selected five products which are sold over the counter for adults and not aimed for treating any specific disease (except vitamin or mineral deficiency). We assumed users took the recommended amount, included all reported sources of each component (e.g. if a subject used both vitamin C tablets and multivitamin we included vitamin C from both sources) and added the median milligram to the amount obtained from nutrition. This resulted in the total (T) vitamin/mineral variables used through the study.

Serum Metabolites

Non-targeted metabolomics profiling analysis was performed for n=855 samples from the control subset of the PopGen. Metabolites in serum samples were extracted with 475 µL methanol and centrifugated. The supernatant was split into 4 aliquots of 100 µL, keeping two as reserve. The other two aliquots were used for LC-MS/MS analysis in positive and negative electrospray ionization mode. LC-MS/MS analysis was performed on a linear ion trap LTQ XL mass spectrometer (Thermo Fisher Scientific, Germany) coupled with a Waters Acquity UPLC system (Waters, Germany). Two different columns (2.1 x 100 mm Waters BEH C18, 1.7 µm particle-size) each optimized for the respective positive or negative electrospray ionization, were used. Metabolites were identified by comparing the recorded LC-MS/MS spectra with spectra found in Metabolon's proprietary spectra library (Metabolon, USA). Further details can be found in Koch et al. (Koch et al., 2017). Of the 855 samples, 400 overlapped with the 1,280 study individuals (LH=228, ObH=145, T2D=27). For each metabolite (totaling 638 in the subset of 400 subjects), the levels measured were divided by the median value of the samples' run day to account for technical day-to-day variations, and missing values were imputed with the minimum detected value.

The ComBat function in R package sva v3.30.0 was applied to correct remaining batch variation, with settings par.prior=F and mean.only = T (Leek et al., 2012). Prior to ComBat, metabolites with zero variance were removed. Among the 390 identified metabolites (based on included standards and manual curation), low-prevalence ("rare") metabolites were separated for handling by appropriate models, specifically those with >50% zero values in non-imputed data, of which a subset of 62 were identified. The remaining 328 identified were considered "abundant".

Microbiome Data from Mouse Iron-Feeding Study

Fecal pellets were transported from UCLA, USA, to Kiel, Germany, frozen on dry ice, processed and 16S sequenced as described for the human samples. The raw 16S data was quality controlled and processed as for the human data to obtain OUT and taxonomic abundance tables for statistical analyses. Following data processing and filtering, 167 samples remained (out of the initial 168).

QUANTIFICATION AND STATISTICAL ANALYSIS

Correcting for Multiple Hypothesis Testing

For all analyses described below and throughout the manuscript, nominal p-values are corrected for multiple hypothesis testing to false discovery rates (q-values) using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Multivariate Linear Modeling Using MaAsLin

For most associations between microbiome and health outcomes we used a modified general linear model as implemented by MaAsLin (Multivariate microbial Association with Linear Models, see for details (Morgan et al., 2012)), which combines an arcsine-square root transformed analysis of relative abundances in a standard multivariable linear model with outlier removal, variable selection by boosting, and (for sparse features) zero inflation. Here, we required a minimum relative abundance of 10^{-10} for taxa and functional features (dMinAbd), and outliers removed >3 inter-quartile ranges past the 1st/3rd quartiles (dOutlierFence).

The full list of covariates to select from in models contrasting healthy and obese subjects (not T2D) were age, gender, fasting glucose levels, total iron intake, antihypertensive and analgesic medications. Only the covariate of health status was enforced. The full list of covariates to select from for models contrasting obese with and without T2D (not lean) were age; gender; BMI; the medications antihypertensives, analgesics, metformin and insulin; and the dietary variables total magnesium and total iron intake. Only the covariates of health status and BMI were enforced. However, to evaluate the effect of metformin/insulin on individual taxa, two models were applied to the taxonomic features that deviate from the above on the specification of metformin/insulin as follows: One without metformin/insulin covariates and one enforcing metformin while keeping insulin and metformin among the full list of covariates. Taxonomic features from 16S data were included as summed at the genus through phylum levels, and with features with >30% zero values were modeled as zero-inflated. The functional features assessed were pathway and gene family group representatives as defined above (using the default model). The specified models were further used to analyze the association between health states and the facultative-to-obligate anaerobe ratio, as well as targeted butyrate metabolizing pathways.

To evaluate the genera associated with obesity in the independent SHIP cohort, MaAsLin was run with same settings across all non-diabetic samples, with the covariates age, gender, antihypertensives and antidepressants (antihypertensives and antidepressants were the most common medications across samples at 49% and 30% users, respectively).

Further, MaAsLin models were used for the analysis of dietary supplement and medication intake. Non-diabetic samples were used except for analyses of antidiabetic medications (insulin and metformin), where only diabetic samples were used. Analyses of medication usage included the covariates age, gender and BMI. For the PopGen and FoCus samples (where dietary supplement data was available) the additional covariates total iron and total magnesium were included. Only the covariates medication and BMI were enforced. Analyses of supplement usage included the covariates age, gender, BMI, analgesic and antihypertensives (used by 22% and 30% of individuals, respectively), enforcing BMI and the evaluated supplement. Finally, MaAsLin was used to analyze associations of microbial taxa and functional processes with age and gender (enforcing those two variables in their respective analyses, as well as health state). Non-diabetic subjects were included and settings were as described above, with covariates selected as in the analysis of obesity.

Variance Explained by Diet and Medication

The function adonis in the R package vegan (Oksanen et al., 2015) was used to calculate the overall variation in microbiome composition and functional capacity that could be explained by both the combined variables, and the variation in microbiome composition that could be explained by any single nutritional variable. Analyses were performed for all non-diabetic subjects. The full list of nutritional variables (n=38), including daily intake from the dietary supplements, is listed in Table S1. The variables diet fibers, proteins, fats, carbohydrates and minerals, were excluded from the analysis of joined variance explained as they are summary measures of the lower-level nutritional variables included in the model. The adjusted coefficient of determination (ω^2) was used as a measure of overall variance explained instead of the coefficient of determination (R^2) to avoid overestimation (Kelly et al., 2015). The coefficient of determination (R^2) was used when evaluating single nutritional variables. We used a permutated null distribution (n=999 samples) of overall variance explained (ω^2) based on reshuffling of sample labels to estimate statistical significance. For calculation of the variance explained by each individual nutritional variable, each variable was analyzed in separate models while adjusting for BMI, age, gender and total energy intake.

Selection of Nutritional Covariates

The inclusion of iron intake in the analysis of both obesity and T2D, and of magnesium intake in analysis of T2D, was based on the difference in number of users and non-users between the health state groups (Table S1), these minerals' overall effects as shown by LDA, and prior knowledge linking them to the respective health states (see main text and Supplemental information). The limited adjustment for nutrient and supplement covariates in the models described above were decided in part based on the analysis of variance: the overall variance explained by dietary intake, including supplements, was estimated at ~1–10% for taxonomic and functional abundance (Figure 3). While these results indicated a slightly greater potential for nutritional data to explain variation in functional capacity (as compared to bacterial abundance), we found that the limited explanatory power argued against inclusion of more dietary variables in the analysis of T2D. As diet is part of the obesity phenotype we also limited dietary covariates in this analysis.

Beta Diversity Association Analysis

To evaluate whether overall bacterial composition or functional capacity associated with obesity (LH versus ObH), BMI, fasting glucose levels or T2D (ObH versus ObT2D), the R function adonis in R package vegan was used with default settings except perm=999, and 16S data at genera level or shotgun metagenomic gene abundance data regrouped to iGO and KO terms, or MetaCyc and GO pathways. To ensure compatible power of the analysis of each trait, data were subsampled to compatible sample sizes (n=100 for metagenomic data, and n=300 for 16S data), and the analyses repeated 50 times to ensure robustness (using the sample function in R with replacement). Furthermore, to ensure compatibility of results, the number of considered covariates were kept at six for all adonis analyses except where diabetic medication was not included: Analysis of obesity and BMI included covariates age, gender antihypertensives, analgesics, total iron intake and fasting glucose levels. Analysis of fasting glucose levels included age, gender, antihypertensives, analgesics, total iron intake and BMI. Analysis of T2D included age, gender, BMI and total iron intake, with or without metformin and insulin as specified with results. Analysis of fasting glucose levels excluded subjects using T2D medication. For the analysis of age and gender, the LH subjects were included, and the analyses were adjusted for covariates as in the MaAsLin-based analysis of obesity. Finally, the function betadisper from the R package vegan was used with default settings to evaluate dispersion between groups of i) LH and ObH and ii) ObH and ObT2D subjects, subsampling and repetitions were used as described for adonis, and the anova function was used to estimate significance.

Alpha-Diversity Association Analysis

Phylogenetic diversity (total unique phylogenetic branch length) was used as the measure of alpha-diversity, and calculated using the phylogenetic tree built on the aligned OTU sequences using FastTree v2 (Price et al., 2010) in nucleotide mode (–nt) and applying a generally time-reversible model (–gtr). The resulting 16S OTU table and phylogenetic tree were used as input in mothur's phylo.diversity function (Schloss et al., 2009). The significance level of the difference in alpha-diversity between groups was estimated using robust regression (function lmRob in R package robust v0.4 (Wang et al., 2017)) adjusting for the covariates listed for MaAsLin.

Medication Association with Microbiome

The importance of medication consumption for bacterial composition (16S data) was evaluated for analgesic, antidepressant, antiphlogistic and anti-diabetic medications in obese T2D samples and for antihypertensives in non-diabetic obese samples. This selection was based on percent medication users within groups and the group with closest to 50% medication users were selected. Individuals were grouped as users or non-users of the target medication classes, and linear discriminant analysis (LDA) was performed as follows; MDS ordination axis were calculated for the relative abundance of genera (16S, excluding genera with zero abundance in all selected samples) using capscale (R package vegan) with Bray-Curtis dissimilarity, and LDA was performed with the first 10 ordination axis, using the MASS package in R. The ordination axes were used instead of the microbial variables directly in order to avoid over-fitting. The obtained linear discriminant function was compared between medication users and non-users with Wilcoxon rank sum test. The same analysis approach was applied to pathway abundance data.

To support the findings in PopGen and FoCus that the gut microbiome could significantly discriminate between users and non-users of five commonly used medication classes, we repeated the LDA with the SHIP data. Analysis of antidiabetics was performed with ObT2D, of antidepressants with ObH and the remaining 3 medication classes (analgesics, antiphlogistics, antihypertensives) were evaluated with data from LH individuals.

Dietary Intake Association with Microbiome
We used LDA as described for medication usage above to evaluate the deviation in microbiome composition between supplement users and non-users of the selected supplements across non-diabetic subjects. We considered age to be the most likely confounding factor in this group of non-diabetic subjects as based on prior findings (see above) and prior publications of microbiome studies. We therefore first considered the distribution of supplement usage across ages and, by visual inspection, found no patterns to prompt the inclusion of age as a covariate.

Serum Metabolites' Association Analysis
For analyses where covariates could not be specified (Mantel, Betadisper), metabolite residuals adjusted for age and gender were used. Residuals were calculated for each metabolite using a linear regression. For all linear models each metabolite was scaled by dividing by the metabolite standard deviation, and the covariates age and gender were included.

Differences in metabolic composition between health states were evaluated using adonis with default settings except method="gower" and perm=10^4, with the covariates age, gender, fasting glucose levels, antihypertensives and analgesics for analysis of obesity, and the covariates age, gender, BMI, metformin, antihypertensives and analgesics for analysis of T2D. Similarly, dispersion between health states was evaluated using betadisper and significance calculated using permutest, both from R package vegan. Correspondence between the metabolite profile and gut microbiome composition (genera) or environment (nutrition, dietary supplements and medication) was evaluated using mantel.rtest in R package ade4 v1.7 (Chessel et al., 2004) across all non-diabetic subjects with metabolite data. Gower distances was used for metabolites and environmental variables to accommodate the mixed data.

The identified and abundant metabolites (n=328) were selected for analysis of single metabolite differences between health states using a linear model in MaAsLin with covariates as above (for adonis), enforcing only the health state and with no data transformation. The remaining 62 metabolites that were identified but with low abundance across the 400 subjects were evaluated for association with obesity or T2D using a chi-squared test (chisq.test function in R package stats) on the scaled, but non-imputed non-adjusted metabolites after conversion to presence/absence data. To obtain information on the direction of the association, e.g. increase or decrease in obese, we applied the Wilcoxon rank sum test in R package stats was used with conf.int=T and the estimate multiplied with -1.

The genera that associated with obesity (q<0.1) were selected and evaluated for association with the 100 abundant metabolites found to associate with obesity. For these, we first tested for an association across all LH samples and then across all obese non-diabetic samples (ObH). The analysis was performed using MaAsLin with covariates age, gender, antihypertensives and analgesics, enforcing only the taxa being evaluated.

Among the 28 metabolites assigned as medication metabolites, 19 metabolites were likely to originate from one of the five medication classes evaluated above. For these 19 metabolites, a generalized linear model with binomial distribution (glm function in R package stats) was applied across all subjects to detect metabolite-to-medication associations. Furthermore, adonis was applied to detect associations between the gut microbiome (genera) and medication metabolite levels (including medication users only and adjusting for health state).

Mouse Features Association with Iron Intake
To evaluate association between overall microbiome composition and iron intake, the microbiome of mice fed 50 ppm and 500 ppm ferrous sulfate were compared. The analysis included data from all time points were mice where on different diets, and was performed using a linear mixed model (function lmer in R package lme4 v1.1 (Bates et al., 2015), with the lmerTest v3.0.1 (Kuznetsova et al., 2017) package to estimate significance and r.squaredGLMM function from R package MuMIn (Bartoń, 2018) to extract variance explained by fixed effect terms). Dissimilarities were calculated for the OTU table using function vegdist with method="bray" in R package vegan. The model included diet (same or different) as fixed effect and intercepts for subjects and cage as random effects to consider dependence between time-points and cage effects, respectively. The same design, however including mice fed chow for the duration of the study, was used to evaluate the dissimilarity between mice on chow versus the respective iron diets.

Comparisons of body composition, glucose tolerance test results, and plasma glucose and insulin levels were performed using the linear mixed model as described above. The area under the glucose curve (AUC) for each mouse was calculated using the formula: AUC = 0.25 (fasting value) + 0.5 (30 minute value) + 0.75 (60 minute value) + 0.5 (120 minute value) per the Schonfeld1 project protocol (https://phenome.jax.org/projects/Schonfeld1/protocol).

R and Additional R-Packages Used
R environment v3.5, RStudio v1.1, grid v3.5 (R Core Team, 2018), gridExtra v2.3 (Baptiste and Antonov, 2017), gridBase v0.4 (Murrell, 2014), gridGraphics v0.3 (Murrell, 2015), reshape2 v1.4 (Wickham, 2007), metaphor v.2.0 (Viechtbauer, 2010), MASS 7.3 (Kafadar et al., 2002), data.table v1.11 (Dowle and Srinivasan, 2019), ggrepel v0.8 (Slowikowski, 2018), plyr v1.8 (Wickham, 2011), dplyr 0,7

(Wickham et al., 2019), stringr v1.3 (Wickham, 2015), caret v6.0, RColorBrewer v1.1 (Neuwirth, 2014), lattice v0.20 (Sarkar, 2008), graphics v3.5 (Murrell, 2018).

DATA AND CODE AVAILABILITY

All data related to the Kiel cohorts (PopGen and FoCus) is available upon application from the PopGen biobank (http://www.uksh.de/p2n/Information+for+Researchers.html). All data related to the Study of Health in Pomerania (SHIP) was obtained from the SHIP data management unit and can be applied for online (https://www.fvcm.med.uni-greifswald.de/dd_service/data_use_intro.php). Data related to the mouse study is available at the NCBI Sequence Read Archive (SRA). The accession number for the mouse data reported in this paper is BioProject: PRJNA550303.

Supplemental Information

Obese Individuals with and without Type

# 2 Diabetes Show Different Gut Microbial

Functional Capacity and Composition

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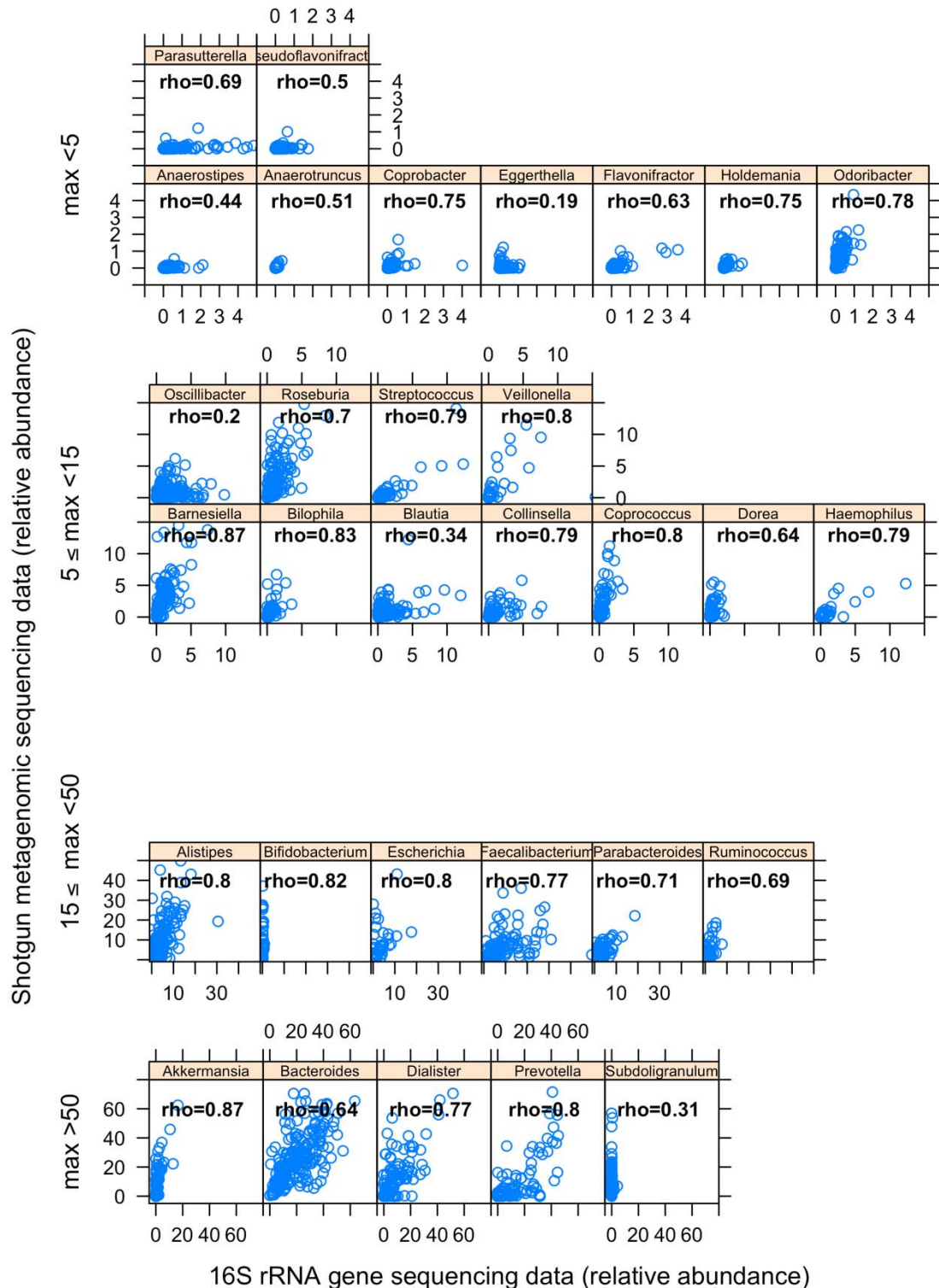
Figure S1: Correlation of 31 genera found in both 16S rRNA gene sequencing data and shotgun metagenomic sequencing data for 201 samples (Related to Figure 1). Genera was separated by max relative abundance in shotgun metagenomic data into four groups as shown on y-axis (cutoff at 5,15 and 50). Shotgun metagenomic data abundance is

illustrated (y-axis) versus 16S rRNA gene abundance (x-axis). Spearman correlation ρ-values are given on each panel (mean ρ=0.67, max=0.87, min=0.19). The genera are also shown on **Figure 1** in a cladogram that illustrates their common ancestry.
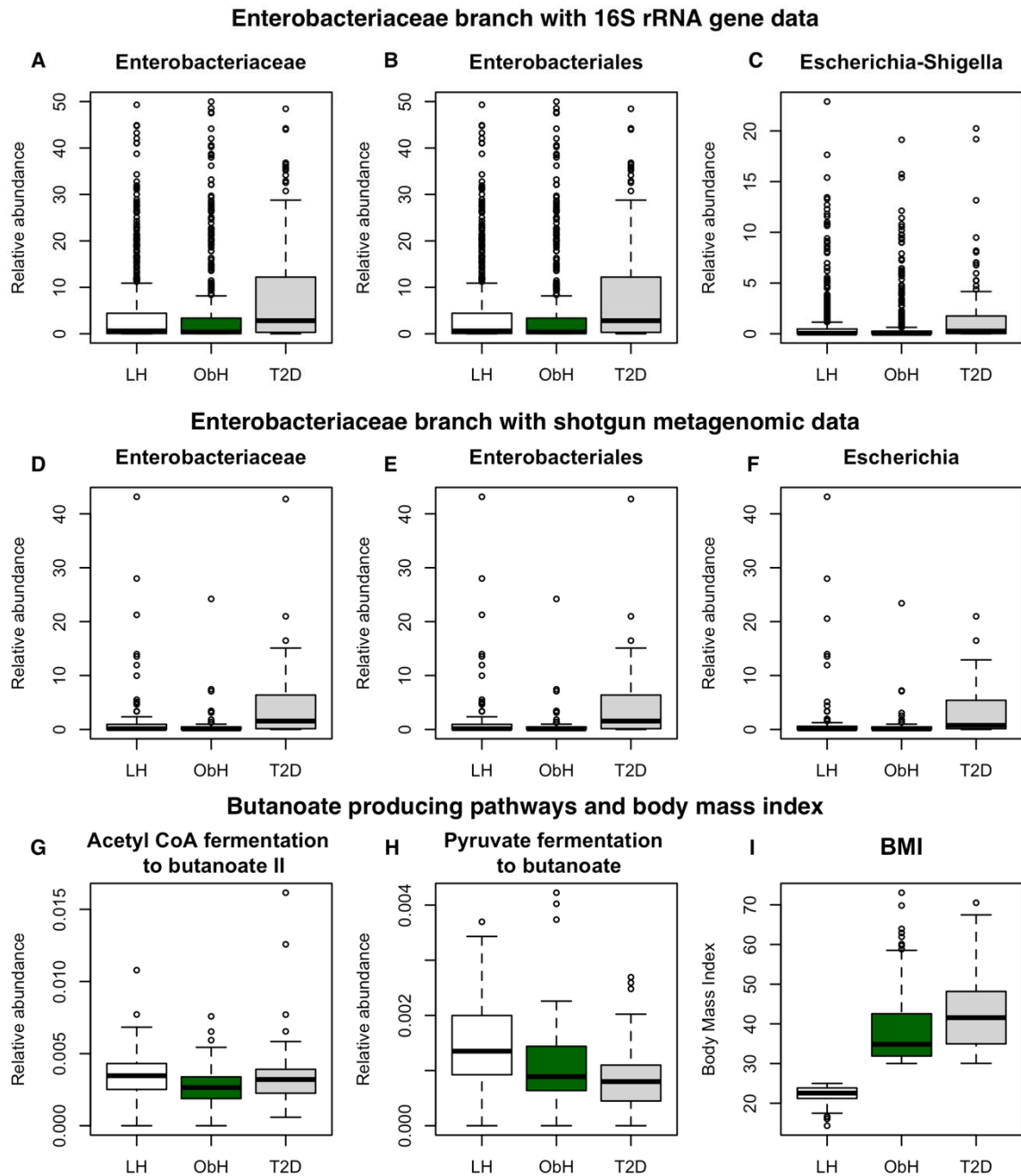
Figure S2: Relative abundance of microbial features and BMI across phenotypic groups (related to Figure 2 and Table S3). The Enterobacteriaceae branch is seen from order to genera level across lean non-diabetic (LH), obese non-diabetic (HOb) and obese individuals with type 2 diabetic (ObT2D) with 16S rRNA gene data (**top panel, A–C**) and shotgun metagenomic data (**mid panel, D–F**). The plots illustrate a higher abundance of this branch of bacteria in the ObT2D samples, and shows that the behavior is consistent

between methods. **G–H)** Relative abundance (as percentage) of two pathways producing butanoate from fermentation of acetyl CoA and pyruvate. We performed targeted analysis of the two main butyrate producing pathways with obesity and T2D in our data (fermentation of acetyl-CoA and pyruvate to butanoate), since our variance and abundance prefilters excluded these pathways from previous analyses (see **Methods**). These pathways showed a decreased abundance in obesity (MaAsLin with covariates as default, q<0.1, LH versus ObH), while there was no difference between obese samples with or without T2D (q>0.1). A more detailed understanding is still needed regarding the relationship between energy harvest and immune signaling in the gut, as it relates to short-chain fatty acids, and the functional association identified here indicate a disturbed butyrate production relates to the metabolic state of obese subjects more than T2D. However, as metformin has been found to both increase the level of SCFA and SCFA-producing bacteria, the power of the presented analysis is reduced by the high number of metformin users among the T2D subjects. **I)** BMI differences between the three health states: lean non-diabetic (LH), obese non-diabetic (ObH), and obese individuals with type 2 diabetes (ObT2D). Default settings are used for the function boxplots in R package graphics.
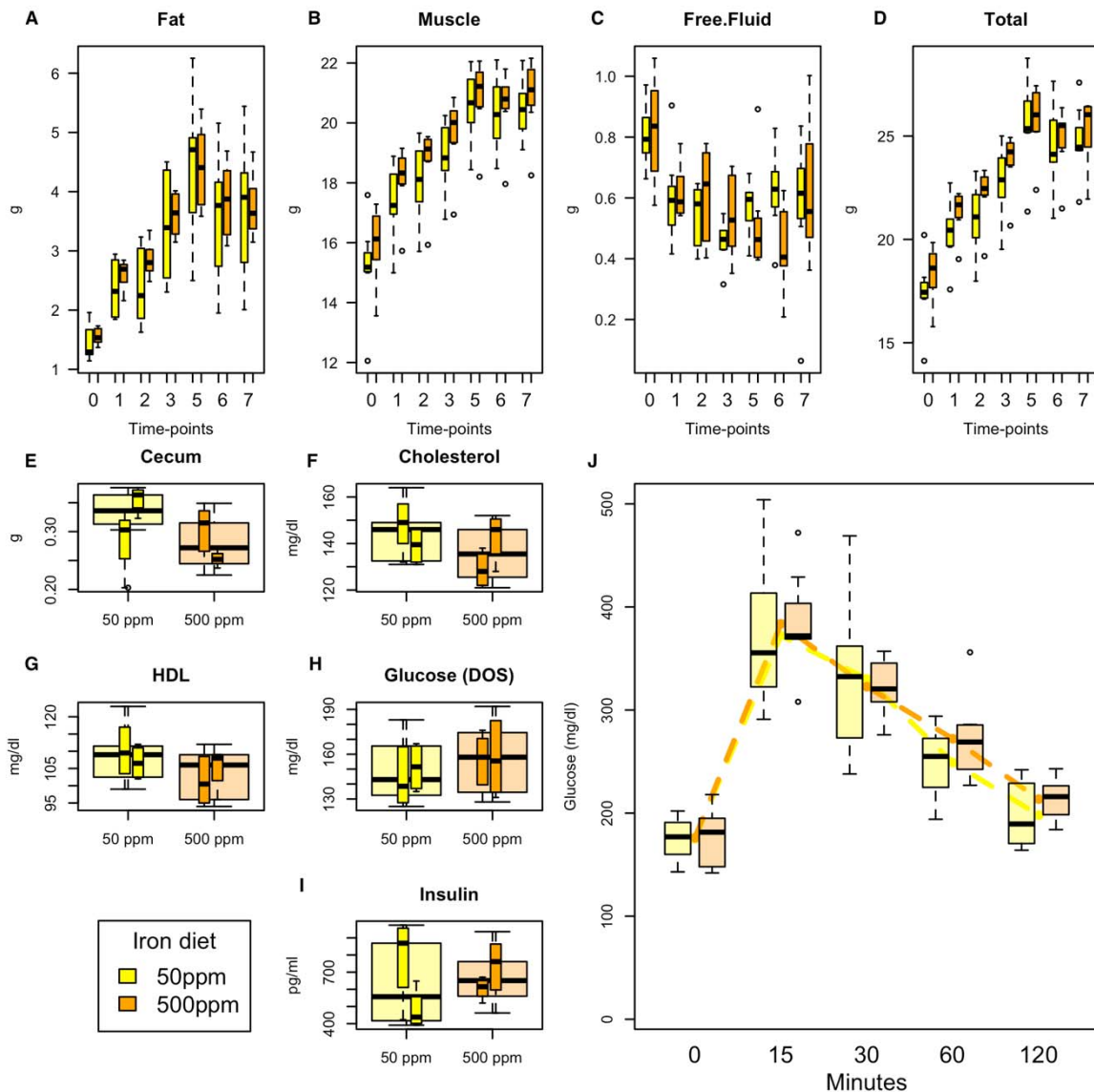
Figure S3: Body composition, blood measures and glucose tolerance of mice on high and low iron diets (relates to Figure 3, Table S6, and STAR methods). Twenty-four male C57BL/6J mice, in groups of 8, were fed one of three diets for a duration of seven weeks: chow, a 50 ppm ferrous sulfate diet or a 500 ppm ferrous sulfate diet. During the seven weeks, extensive evaluation of their body composition and blood measures was performed. **A-D)** show the development of fat, muscle, free fluid and total body mass (gram) over the study duration (x-axis for seven time points), including baseline (TP0) where all mice where kept on chow. Yellow boxes represent mice on 50ppm iron and orange boxes represent mice on 500ppm iron diets. Visual inspection indicated a trending increase in total body weight and muscle mass in mice on high iron diets, however statistical evaluations of the body composition measures between iron intake groups showed no significant differences (linear mixed model, q>0.1, **Table S6**). Free fluid mass decreased in both diet groups when first changed to iron diets, but then by visual inspection appeared to continue the decreased more in mice on high iron as compared to low iron diets (Figure S3), however there was no significant difference between the iron groups when evaluated across all six time-points with different diets. **E-I)** show results for the measurements of cecum weight, and levels of

cholesterol, HDL, glucose and insulin at the end of the study. Broad boxes show data across all eight mice in each diet group, and these boxes are overlaid with boxes showing data for mice in each cage (four mice per cage). Neither cecum weigh, nor any of the measurements of cholesterol, HDL, glucose or insulin at the end of the study duration was significantly different between the iron groups (q>0.1, **Table S6**). **J)** To further evaluate if the iron diets associated with physical differences related to obesity or diabetes, a glucose tolerance test (GTT) was performed after four weeks on the study diets. The plot shows the glucose levels pre-injection (0 minutes) and at four time-points following glucose injection. Boxplots are shown at each time-point, as well as a line for each diet group (yellow for 50 ppm, orange for 500 ppm) showing the development in the mean glucose levels.
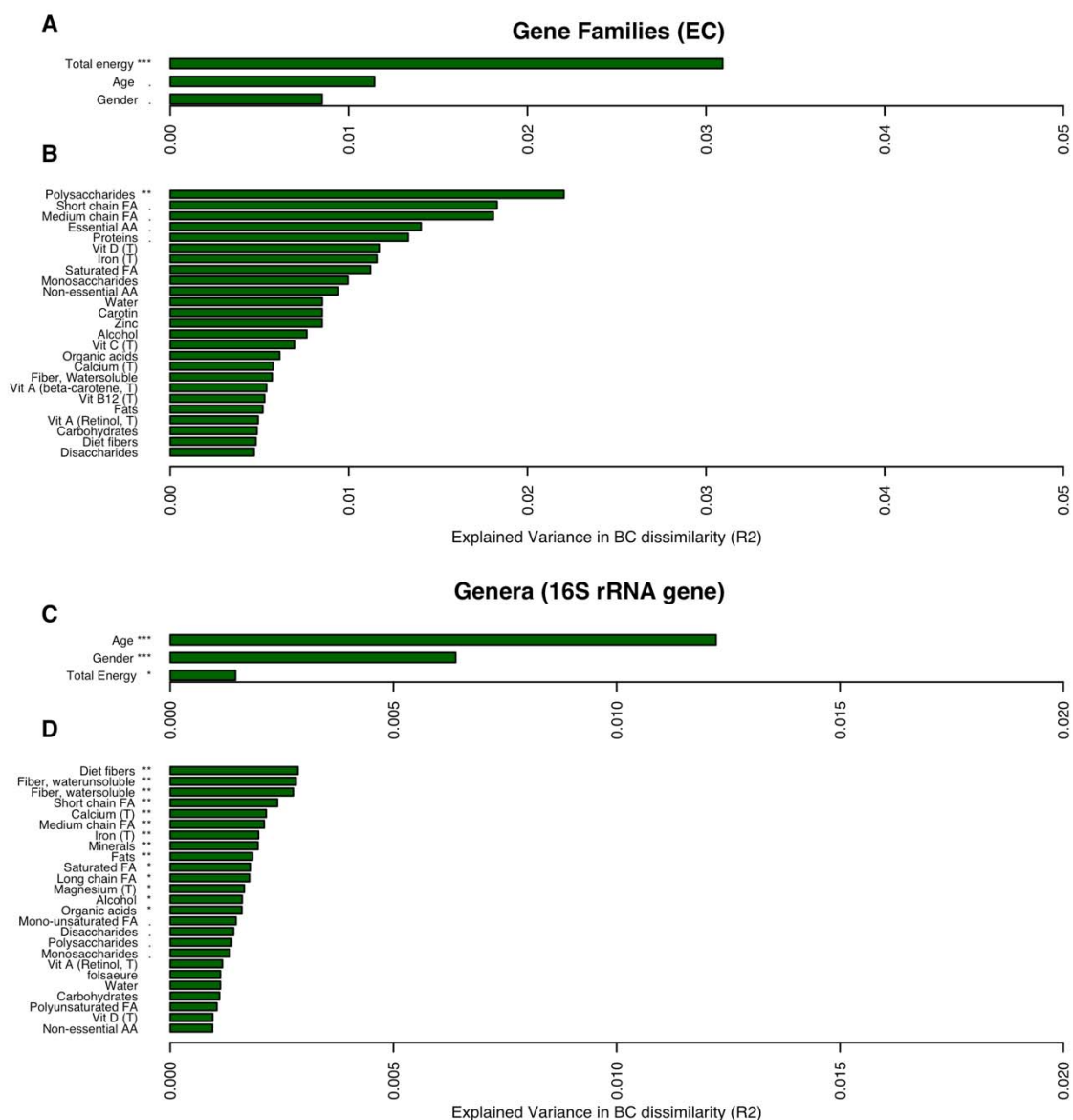
Figure S4: Long-term nutritional variables explain limited variation in microbiome composition and functional capacity (related to Figure 3 and Table S1). Variance explained by single variables in **A-B)** gene families (metagenomes) and **C-D)** genera (16S). The variance explained (R^2) calculated using adonis (in R package vegan) is shown for age, gender and total energy intake (**A** and **C**), and single nutrients, including dietary

supplements after adjusting for BMI, age, gender and total energy (**B** and **D**). Included variables were selected based on R^2 values as top 25 variables from each analysis. The 'T' listed with a subset of variables indicate these variables are the joined intake from nutrients and dietary supplements. Significance of BH-adjusted p-values is indicated as follows: '***' for q<0.01, '**' for 0.01≤q<0.05, '*' for 0.05≤q<0.1, '.' for 0.1≤q<0.2. FA: Fatty acids, AA: Amino acids. See overview of nutritional variables in **Table S1**.