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Effects of underfeeding and oral vancomycin on gut microbiome and nutrient absorption in humans

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Direct evidence in humans for the impact of the microbiome on nutrient absorption is lacking. We conducted an extended inpatient study using two interventions that we hypothesized would alter the gut microbiome and nutrient absorption. In each, stool calorie loss, a direct proxy of nutrient absorption, was measured. The first phase was a randomized cross-over dietary intervention in which all participants underwent in random order 3 d of over- and underfeeding. The second was a randomized, double-blind, placebo-controlled pharmacologic intervention using oral vancomycin or matching placebo (NCT02037295). Twenty-seven volunteers (17 men and 10 women, age 35.1 ± 7.3, BMI 32.3 ± 8.0), who were healthy other than having impaired glucose tolerance and obesity, were enrolled and 25 completed the entire trial. The primary endpoints were the effects of dietary and pharmacological intervention on stool calorie loss. We hypothesized that stool calories expressed as percentage of caloric intake would increase with underfeeding compared with overfeeding and increase during oral vancomycin treatment. Both primary endpoints were met. Greater stool calorie loss was observed during underfeeding relative to overfeeding and during vancomycin treatment compared with placebo. Key secondary endpoints were to evaluate the changes in gut microbial community structure as evidenced by amplicon sequencing and metagenomics. We observed only a modest perturbation of gut microbial community structure with under- versus overfeeding but a more widespread change in community structure with reduced diversity with oral vancomycin. Increase in *Akkermansia muciniphila* was common to both interventions that resulted in greater stool calorie loss. These results indicate that nutrient absorption is sensitive to environmental perturbations and support the translational relevance of preclinical models demonstrating a possible causal role for the gut microbiome in dietary energy harvest.

The global obesity epidemic has inspired efforts to identify both environmental and host factors influencing energy balance, defined as the equilibrium between energy intake and expenditure[1–3]. While calorie consumption is a key determinant of energy intake, the extent to which individuals vary in their ability to digest and absorb consumed dietary substrates may also impact energy balance. Human data show that energy loss in stool can vary from ~2–9% of ingested calories[4,5]; however, the mechanisms that contribute to this variation and their physiological relevance remain poorly understood. In contrast, an abundance of mechanistic insight has been generated over the past decade using rodent models, supporting a causal role of the trillions of microorganisms that colonize the human gastrointestinal tract (the gut microbiota) in shaping interindividual variations in energy balance, due to their broad impacts on both energy intake and expenditure[6–10].

Multiple studies support the translational relevance of the gut microbiota for human energy balance. A comparison of lean and obese subjects provided with a fixed calorie diet (3,400 kcal d^{-1} and 2,400 kcal d^{-1}) revealed that underfeeding (UF) significantly increases stool calorie loss and decreases the relative abundance of the Firmicutes relative to the Bacteroidetes[5], the two dominant phyla in the human gut[11]. In lean individuals, decreased Firmicutes relative to Bacteroidetes was associated with stool calorie loss[5]. These rapid changes (within days) in gut microbial community structure and the association of these changes with stool calorie loss in the lean cohort are consistent with the hypothesis that the human gut microbiota affects nutrient absorption, yet causal evidence from human populations are still lacking. Attempts to transplant the gut microbiota of lean individuals into obese recipients have not significantly altered body weight or adiposity; however, there were improvements in insulin sensitivity and other markers of lean body weight[12,13]. The energetic consequences of pharmacologic perturbations of the microbiome in the form of antibiotic administration appear to be context dependent. Early life antibiotic use is associated with greater childhood weight gain[14] and there is mixed evidence for antibiotic-induced growth promotion in malnourished

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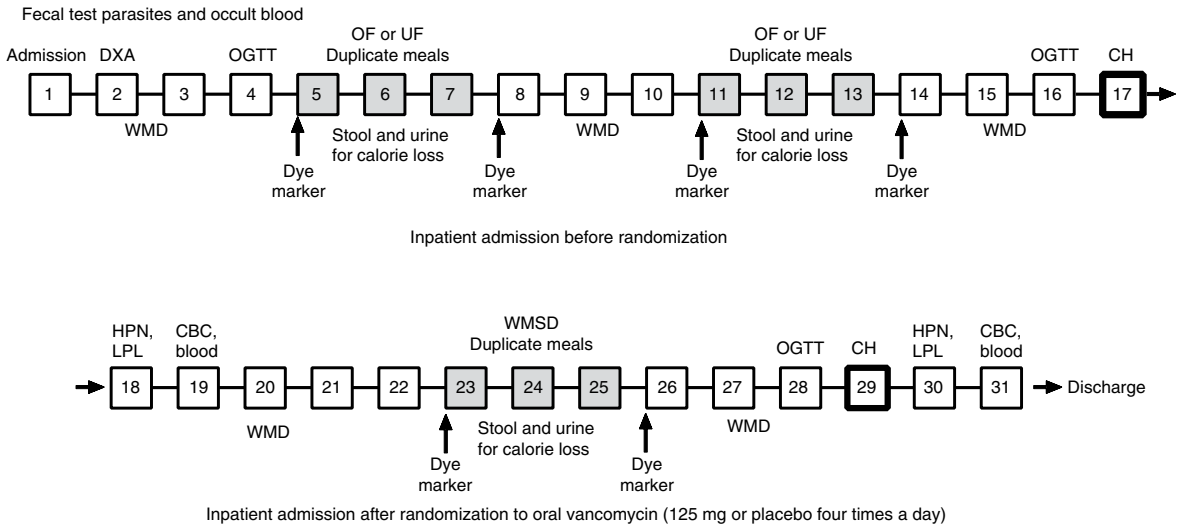


Fig. 1 | Study outline. Following screening, participants were admitted and placed on a WMD. Dual-energy X-ray absorptiometry (DXA) scan was performed on day 2 and OGTT on day 4 of admission. Participants were then fed (in random order) 50% of WMD (UF) and 150% of WMD (OF) on days 5–7 and 11–13, with a 3-d washout period in between. Dye marker for stool collection was given on the mornings of days 5, 8, 11 and 14. Collection of food (duplicate meals to perform bomb calorimetry) was done on days 5–7 and 11–13. Following the UF/OF phase, participants were on WMD for 2 d then on consecutive days underwent OGTT (day 16) measurement of 24-h energy expenditure in a whole-room indirect calorimeter (day 17) and measurement of fasting ANGPLT4 and heparin-induced measurement of LPL (day 18). Following this last test, participants were then randomized to oral vancomycin versus placebo. On days 23–25 while on WMD, meals were again made in duplicate for bomb calorimetry. Participants again ingested dye marker to mark stool on the morning of day 23 before breakfast and again on the morning of day 26. Stool and urine were collected between dye markers for measurement of calorie excretion. Whole-room indirect calorimetry for the assessment of the 24-h energy expenditure and substrate oxidation was repeated on day 29; heparin-induced measurement of LPL and ANGPTL4 was repeated on day 30. CBC, complete blood count; CH, respiratory chamber ; HPN, heparin bolus; WMSD, weight-maintaining special diet.

children[15,16]. In adults, a 7-d course of oral antibiotics failed to elicit any significant differences in stool calorie loss despite widespread changes in gut microbial community structure and function[17]. These negative results are surprising given the extensive data in animal models, wherein microbiota depletion with antibiotics or housing under sterile (germ-free) conditions results in marked differences in metabolic phenotypes of adult animals[6–8].

Taken together, the causal evidence from multiple animal model studies and largely correlative or retrospective data from human cohorts led us to further investigate the role of the human gut microbiome in nutrient absorption. We designed a clinical study (ClinicalTrials.gov identifier: NCT02037295) to test whether interventions designed to alter the gut microbiota cause significant differences in stool calorie loss, which serve as an experimentally quantifiable proxy for nutrient absorption. In this two-phase study, we directly measured stool and ingested calories along with plasma biomarkers of host and microbial metabolism during overfeeding (OF) and UF (phase I) and then in a randomized, double-blind, placebo-controlled trial with oral vancomycin versus placebo (phase II). Previous studies have indicated that oral vancomycin can decrease the relative abundance of the Firmicutes phylum while preserving Lactobacillaceae[18] which has been associated with beneficial metabolic effects[19]. Oral vancomycin also has minimal absorption and thus nearly no local or systemic side effects in healthy individuals[20].

Results

UF and antibiotics both impair nutrient absorption. We conducted a controlled dietary study in two contiguous phases (Fig. 1). Individuals were admitted to the Clinical Research Unit (CRU) for approximately 31 d (Fig. 1), during which they were fed a weight-maintaining diet (WMD) (20% kcal protein; 30% kcal fat; 50% kcal carbohydrate) adjusted to maintain a stable weight (±1%). In total, we screened 51 potential participants, of which 27 initiated and 25 completed the study (Extended Data Fig. 1). In phase I, participants were fed 3 d each of 150% of WMD (OF diet) and 50% of WMD (UF diet) with a 3-d washout period in between in random order in a cross-over design. In phase II, the same subjects were randomized to placebo or oral vancomycin. There were no significant differences in the vancomycin versus placebo groups in sex, age, body weight, body mass index (BMI), percentage body fat or glucose homeostasis (Supplementary Table 1). Participants were inpatients for the entire study and were monitored daily, and there were no reported side effects, in particular diarrhea or abdominal symptoms.

Our results from phase I demonstrated that caloric intake led to significant differences in nutrient absorption. As specified by our study design, daily ingested calories were significantly higher during OF ($4{,}446.5 \pm 547.8\,\mathrm{kcal\,d^{-1}}$) relative to UF ($1{,}494.2 \pm 211.0\,\mathrm{kcal\,d^{-1}}$; Fig. 2a). On average, this corresponds to a difference of $2{,}952.3\,\mathrm{kcal\,d^{-1}}$. Food and stool calories were measured by bomb calorimetry, allowing us to calculate percentage calorie loss for each individual ($100 \times$(stool calories/ingested calories)). As expected, the absolute number of calories lost per day was significantly higher in overfed relative to underfed individuals ($257.4 \pm 91.2\,\mathrm{kcal\,d^{-1}}$ versus $123.5 \pm 32.4\,\mathrm{kcal\,d^{-1}}$, $\Delta = 139.4\,\mathrm{kcal\,d^{-1}}$, $P < 0.0001$, paired Student's t-test; Fig. 2b and Supplementary Table 2). However, when expressed as percentage of ingested calories per day, there was significantly greater stool calorie loss during UF relative to OF ($8.9 \pm 3.7\%$ versus $5.8 \pm 1.9\%$, $\Delta = 2.3\%$ per day, $P < 0.0001$, paired Student's t-test; Fig. 2c and Supplementary Table 2), with a strong and significant intrasubject correlation (Pearson's $r = 0.70$, $P = 0.004$). Transit time was faster during UF compared with OF ($\Delta = -10.6$ h, $P = 0.0035$, paired Student's t-test; Fig. 2d). Daily calorie loss from urine was lower during UF versus OF in absolute values (Supplementary Table 2) but was higher during UF versus OF when expressed as percentage of ingested calories (Extended Data Fig. 2a and Supplementary Table 2). There was no detectable effect of diet order or sex (all $P > 0.07$; Supplementary Table 3).

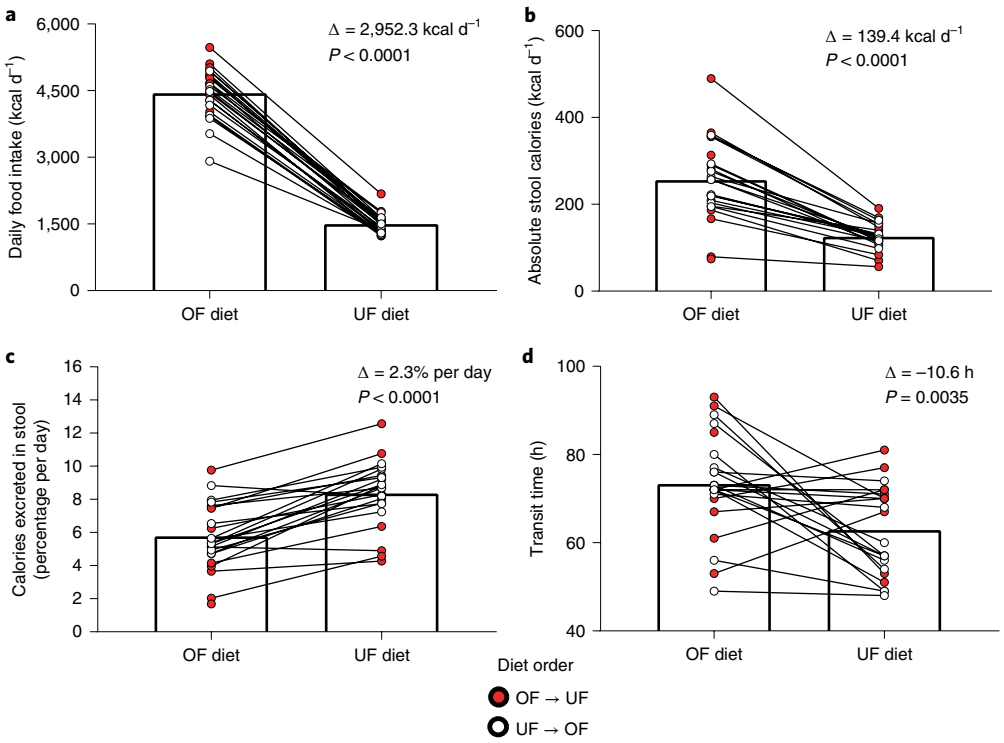


Fig. 2 | Dietary interventions impact nutrient absorption. Graphs show results from 23 participants who completed the UF and OF phase of the study. Two participants did not have available stool measurements during UF. **a**, Mean of 3-d food intake during OF ($n=25$) and UF ($n=25$) diets, expressed in kilocalories per day. **b**, Mean stool calories expressed as absolute calorie loss (kcal d^{-1}) during OF ($n=25$) and UF ($n=23$). **c**, Mean stool calories expressed as percentage excretion, calculated as (stool calories (kcal d^{-1})/ingested calories (kcal d^{-1})) × 100 during OF ($n=25$) and UF ($n=23$) diets. **d**, Mean transit time, expressed in hours, during OF ($n=25$) and UF ($n=23$). Δ indicates the mean difference between groups (UF versus OF). *P* values determined by two-sided paired Student's *t*-test. Participants who started the dietary phase with OF are represented by red dots, while those who started with UF are represented by white dots. The transit time was defined as the time between the first and the last appearance of the dye marker during each intervention, in hours.

Next, we sought to compare these results in a parallel study design aimed at directly targeting the gut microbiota. As specified by our study design, daily ingested calories were comparable between the placebo and vancomycin groups (Fig. 3a and Supplementary Table 2). Vancomycin led to a significant increase in stool calorie loss expressed in absolute kilocalories per day (260.1 ± 66.5 kcal d^{-1} versus 176.8 ± 79.5 kcal d^{-1}, Δ = 83.2 kcal d^{-1}, $P=0.012$, unpaired Student's *t*-test; Fig. 3b) or the percentage of ingested calories (8.4 ± 1.9% versus 5.8 ± 2.2%, Δ = 2.6, $P=0.0069$, unpaired Student's *t*-test; Fig. 3c). Importantly, transit time did not differ between vancomycin and placebo groups ($P=0.2$, unpaired Student's *t*-test; Fig. 3d). In contrast to the dietary intervention, there was no difference in urine caloric excretion between the vancomycin and placebo groups (Extended Data Fig. 2b and Supplementary Table 2). No differences were observed when the analysis was stratified by sex (Supplementary Table 4). There was also no absolute change in trajectory for body weight in individuals treated with vancomycin (Δ = 0.2 kg, $P=0.5$, unpaired Student's *t*-test). Taken together, these results support our hypothesis that both UF and vancomycin result in greater stool calorie loss relative to ingested calories (greater percentage of calories measured in stool), indicating a reduction in nutrient absorption in both states.

Shifts in the gut microbiome in response to UF and oral antibiotics. To identify candidate effector microorganisms that could contribute to the observed differences in nutrient absorption, we evaluated the impacts of dietary and pharmacological perturbations on gut microbial community structure using a combination of amplicon and metagenomic sequencing. We sequenced samples from all 25 participants at key time points during phase I, including a baseline sample collected at the beginning of the trial, midpoint samples collected during each 3-d UF and OF period, samples collected pre and post each dietary intervention and three samples collected during the vancomycin or placebo arm (Supplementary Table 5). To examine longitudinal changes in gut microbial structure throughout the study, we also sequenced daily samples during phase I (16 d) for ten subjects, including five subjects who showed the highest difference in percentage stool calories loss and another five subjects who showed the lowest difference in percentage stool calories loss between the dietary arms (Supplementary Table 5).

Our nutritional results from phase I (Fig. 2) led us to speculate that the gut microbiota may be relatively 'starved' of nutrients during the UF phase, which would be expected to lead to a decrease in bacterial colonization. Surprisingly, we found a significant increase in total colonization levels (Fig. 4a) during UF versus OF. These differences were still significant after normalizing for stool production ($P=0.005$, paired Wilcoxon test). Overall microbial community structure was maintained throughout the 3-d UF and OF interventions, maintaining the significant interindividual differences at baseline (Fig. 4b–d). When analyzed within each subject, we were able to identify two individuals (of ten evaluated) that had significant differences in overall microbial community structure between diet arms (Fig. 4e), which may suggest that the baseline gut microbiota matters for its sensitivity to the dietary interventions. Overall microbial diversity was also relatively stable throughout the 16 d of phase I (Fig. 4f); however, there was significant increase in diversity on the final day of UF relative to the final day of OF ($P=0.04$, paired Wilcoxon test). There were no significant differences

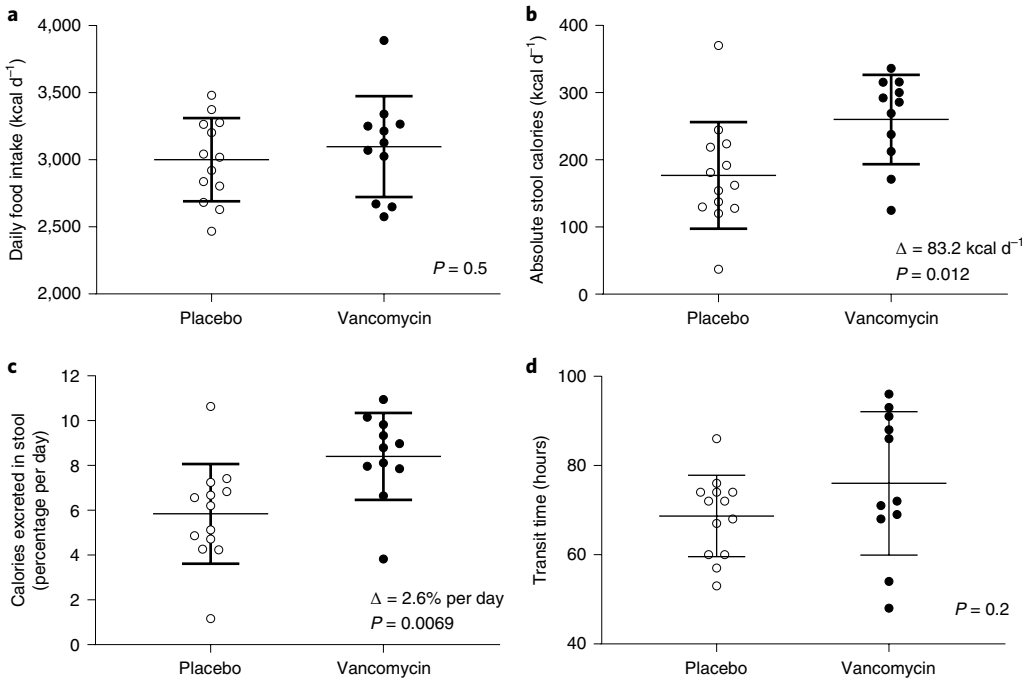


Fig. 3 | Pharmacologic interventions impact nutrient absorption. Results from 24 participants who completed the randomized phase of the study. One volunteer in the vancomycin group did not have available stool measurements. **a**, Mean of 3-d food intake (kcal d^{-1}) in the vancomycin ($n=11$) and placebo ($n=13$) groups. **b**, Mean absolute calorie loss in stool (kcal d^{-1}) in the vancomycin ($n=11$) versus placebo ($n=13$) groups. **c**, Mean percentage calorie loss in stool (kcal d^{-1}) in the vancomycin ($n=11$) versus placebo ($n=13$) groups. **d**, Mean transit time, expressed in hours, in vancomycin ($n=11$) and placebo ($n=13$) groups. Δ indicates the mean difference between groups (vancomycin and placebo). *P* values determined by two-sided unpaired Student's *t*-test. Transit time was defined as the time between the first and the last appearance of the dye marker during each intervention, in hours. Error bars represent the mean with 95% confidence interval.

in phylum- or genus-level relative abundance between diet arms, with the exception of the Verrucomicrobia phylum which was significantly increased during UF (Extended Data Fig. 3). At a finer taxonomic level, we were able to identify four 16S ribosomal RNA sequence variants that were significantly associated with the diet arm (independent of order): *A. muciniphila*, *Bacteroides coprocola*, a *Lachnospiraceae* sp. and a *Ruminoccoccus* sp. (Fig. 4g). Taken together, these results indicate the overall resiliency of the gut microbiota faced with differences in caloric intake while also highlighting a small number of diet-responsive bacterial species that could contribute to the observed differences in nutrient absorption.

Next, we used metagenomic sequencing to confirm and extend the observed shifts in gut bacterial relative abundance. Samples were selected from 18 individuals at four key time points including a baseline sample, midpoint samples during each 3-d UF and OF period, a sample during the washout period in between and a sample during the vancomycin or placebo arm. In total, we generated 381.7 giga base pairs of sequencing data from the 95 samples during phase I (Supplementary Table 6). Differentially abundant bacteria on each diet arm (independent of order) were identified using a paired Wilcoxon test. This analysis revealed nine bacterial species that were significantly different between the two diet arms (Fig. 4h and Supplementary Table 7), including an enrichment for *A. muciniphila* during UF, consistent with our 16S rRNA gene sequencing-based analysis (Fig. 4g). We also detected an enrichment for multiple *Alistipes* species during UF.

In contrast to caloric intake, the oral antibiotic vancomycin had dramatic and reproducible impacts on the gut microbiota relative to placebo controls. While colonization level was not different by treatment group ($P=0.26$, unpaired Wilcoxon test; Fig. 5a), there was a significant decrease in microbial diversity, as assessed by 16S rRNA gene sequencing ($P<0.0001$, unpaired Wilcoxon test; Fig. 5b). Vancomycin also had a significant impact on gut microbial community structure, outweighing the pre-existing interindividual differences (Fig. 5c). Compared with placebo, the vancomycin-treated subjects had marked changes in phylum-level relative abundance (Extended Data Fig. 4a). Finer-level analysis of sequence variants demonstrated several discrete clusters of bacteria across different phyla that were altered in the vancomycin-treated group (Extended Data Fig. 4b and Supplementary Table 8).

Metagenomic sequencing confirmed the broad and reproducible differences between the vancomycin and placebo groups. Analysis using unpaired Wilcoxon tests revealed 41 bacterial species with a significant difference (false discovery rate (FDR) < 0.05) in relative abundance (Fig. 5d), with a skew towards decreased abundance in the vancomycin group (31 decreased, 10 increased). Consistent with our 16S rRNA gene sequencing data, these vancomycin-depleted bacteria spanned multiple gram-positive (Actinobacteria and Firmicutes) and gram-negative (Bacteroidetes) phyla. Three *Veillonella* species[21] were significantly enriched in vancomycin-treated individuals. We also detected an enrichment for multiple *Lactobacillus* and *Klebsiella* species in the vancomycin group, in addition to a virulent bacteriophage that infects lactobacilli (Lc-Nu)[22], consistent with evidence in mouse models that antibiotics induce phage production[23].

Despite increased stool calorie loss with both UF versus OF and vancomycin versus placebo, we found markedly different effects of these interventions on the gut microbiota. UF and OF produced little perturbation at the phylum or finer levels, while vancomycin resulted in widespread changes in gut microbial community structure. Despite these differences, we were able to identify two bacterial species that consistently changed in response to both perturbations (Fig. 5e) based on 16S rRNA sequence variants analysis: *A. muciniphila*, a mucin-degrading bacterium with a putative role in preserving gut barrier

function[24–26], which was enriched during UF and in the vancomycin group, and an unidentified species belonging to *Lachnospiraceae* NK4A136 group, which decreased during UF and vancomycin interventions. Metagenomic sequencing confirmed the enrichment for *A. muciniphila* during UF (Fig. 4h), and a trend towards increased *A. muciniphila* abundance in the vancomycin group ($P=0.25$).

Mechanistic insights into host–microbiome interactions relevant to metabolic disease in UF versus OF and vancomycin versus placebo interventions. To gain further insight into the specific bacterial genes, enzymes and metabolic pathways associated with nutrient absorption, we annotated our metagenomic datasets using HUMAnN2 (ref. [27]). Consistent with our taxonomic analyses (Fig. 4), which revealed modest perturbation of gut microbial community structure with UF versus OF, functional profiling of the microbiome datasets did not reveal any significant differences in gene family or metabolic pathway abundance between the diet arms. In contrast, we found significant differences in the abundance of 760 of a total of 3,553 Kyoto Encyclopedia of Genes and Genomes (KEGG) functional orthologs (KEGG Orthology) (FDR < 0.1, Supplementary Table 9) between vancomycin and placebo, consistent with the widespread changes in gut microbial community structure (Fig. 5a–d). At the pathway level there was a more modest difference between groups; only 3 of 290 metabolic pathways were differentially abundant (all decreased) with vancomycin compared with placebo (Extended Data Fig. 5). Interestingly, two of the three significant pathways (pyruvate fermentation to butanoate and the superpathway of *Clostridium acetobutylicum* acidogenic fermentation) were related to the fermentation of sugars to the major short-chain fatty acid butyrate (aka butanoate), suggesting that there could be reduced butyrate production or bacterial metabolism by the gut microbiota during vancomycin treatment.

To provide more direct support for the inferred differences in gut microbial metabolism, we evaluated plasma short-chain fatty acids as markers of the ability of the gut microbiome to process and metabolize nutrients, together with hormones and bile acids implicated in nutrient absorption and gut barrier function. These measurements were performed before and following each intervention. Consistent with earlier studies[28–33], GLP-1 and leptin significantly increased during OF and leptin and GLP-2 significantly decreased during UF (Supplementary Table 10). Butyrate, a major end-product of gut bacterial metabolism, was significantly decreased following both UF (Fig. 6a) and vancomycin (Fig. 6b), supporting our metagenomic analyses and consistent with a decrease in the capacity of the gut microbiome to harvest nutrients during these interventions. Deoxycholic acid, a secondary bile acid, also significantly decreased following both interventions (Fig. 6c,d). Elevated deoxycholic acid has been implicated in increased gut permeability[34]. Lower concentrations may imply preservation of the gut barrier and lower nutrient absorption. Vancomycin also decreased acetate, propionate and the secondary bile acid lithocholic acid (Extended Data Fig. 6), consistent with the broad impact of this oral antibiotic on the gut microbiome.

Increased stool calorie loss might be due to improved gut barrier function, thereby decreasing gut permeability. We measured two circulating plasma markers that when increased may signify increased gut permeability: zonulin, a modulator of intercellular tight junctions[35]; and lipopolysaccharide (LPS), a product of gram-negative bacteria[36]. There were no changes in plasma zonulin or LPS concentrations with UF (Supplementary Table 10). During OF, plasma zonulin concentration increased ($\Delta=0.4$, $P=0.04$, paired Student's *t*-test) but there was no change in plasma LPS concentrations. Plasma LPS and zonulin concentrations did not change during vancomycin or placebo administration. Except for a slight increase in zonulin with OF, we did not find evidence of altered gut permeability. Lastly, we evaluated whether vancomycin administration affected energy or substrate oxidation rates or metabolites that regulate fat storage and glucose metabolism. There were no significant differences in (1) angiopoietin-like 4 (ANGPTL4) or lipoprotein lipase activity (LPL) (Supplementary Table 11); (2) 24-h energy expenditure or substrate oxidation (Extended Data Fig. 7); or (3) glucose tolerance or insulin concentrations (Extended Data Fig. 8).

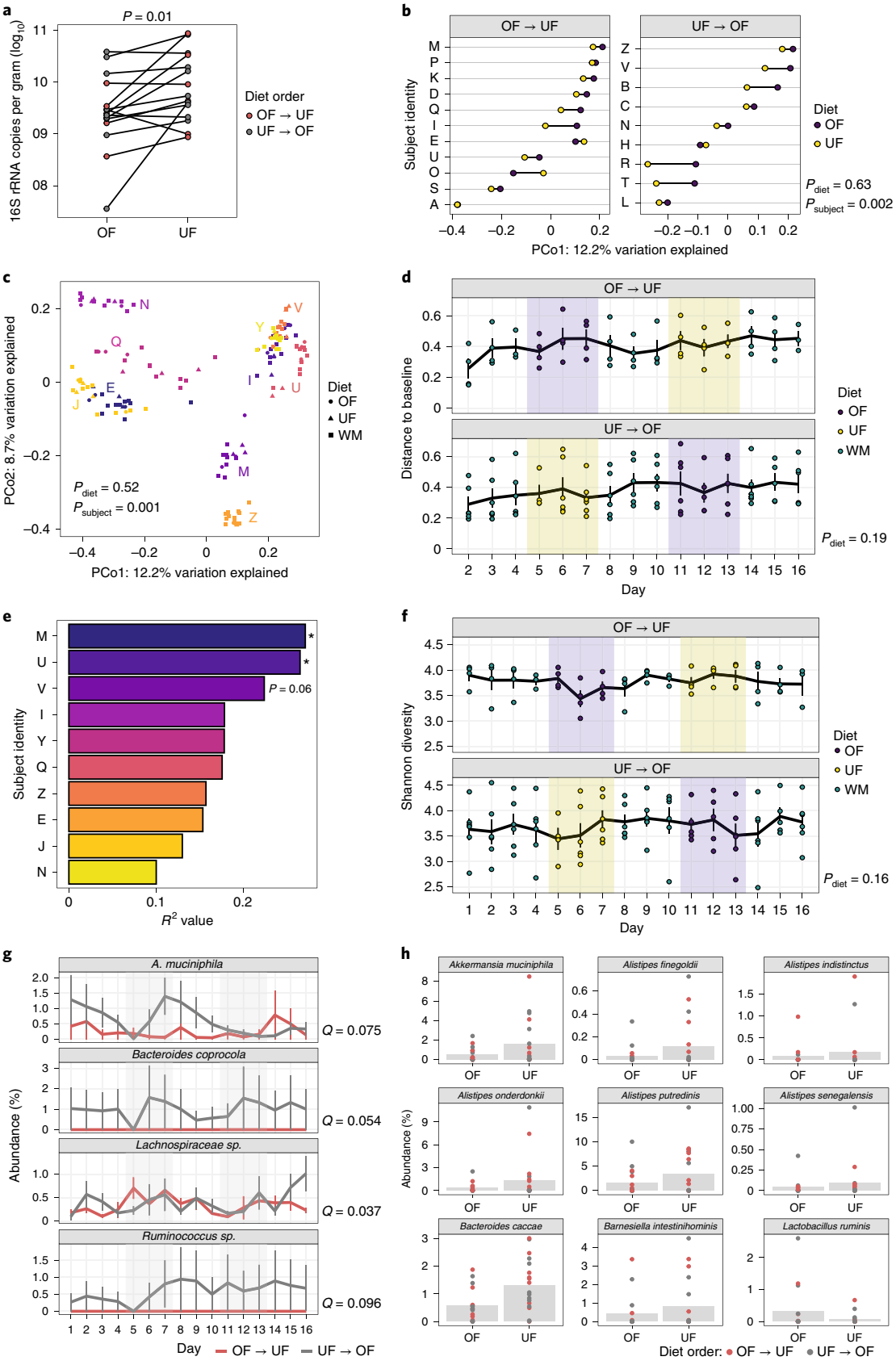
Discussion

In this two-phase study we directly measured ingested and stool calories, demonstrating an increased and comparable stool calorie loss relative to ingested calories during two states: UF versus OF and oral vancomycin versus placebo. This increase in stool calorie loss during UF and vancomycin administration occurred despite different effects on the gut microbiota. We saw a modest perturbation of gut microbial community structure with UF versus OF, while oral vancomycin induced widespread changes with reduced diversity and marked shifts in gut bacterial relative abundances. Decreased plasma butyrate and deoxycholic acid concentrations were common to both interventions that induced greater stool calorie loss. Our study employs direct measurements of ingested, stool and urine calories and close monitoring of and consistency of dietary intake, through the critical periods of stool collection. As participants were inpatients, provided stool samples were timed so that we were able to analyze gut bacterial abundance concurrent with interventions.

As a major end-product of bacterial metabolism, plasma butyrate is a marker of the availability or ability of the gut microbiota to process nutrients[37]. Given this, our results indicate that either decreased calorie delivery to the microbiome, as in the case of UF, or widespread changes in its structure, due to oral vancomycin, limit microbiome nutrient harvesting capacity, resulting in increased stool calorie loss. This offers evidence based on directly measured calories, in the form of stool calorie measurement accounting for ingested calories, that in humans the gut microbiome has a measurable role in metabolizing nutrients. Based on our results, we would estimate that the gut microbiome harvested an additional ~2.5% of absolute ingested calories under the conditions used for our study cohort.

Fig. 4 | Changes in gut microbiome in response to dietary interventions. a, Overall gut microbial colonization during OF and UF as demonstrated by qPCR-based quantification of 16S rRNA gene copies per gram wet weight ($n=30$ samples from 15 individuals; two-sided paired Wilcoxon test). **b**, First principal coordinate of Bray–Curtis dissimilarity between the two diet arms. Subjects are split by diet order ($n=11$ OF → UF and $n=9$ UF → OF). **c**, First two principal coordinates of Bray–Curtis dissimilarity for daily samples from ten subjects during the dietary phase. Each point represents a single sample ($n=156$ samples), where color denotes subject identity and shape denotes diet (weight maintaining, WM). **d**, Distance of microbial communities to baseline sample (day 1) over time. Colors indicate diet; each point represents a single sample ($n=156$ samples). Subjects are split by diet order with OF (purple) and UF (yellow) periods shaded. Lines represent mean ± s.e.m. *P* value, two-sided paired Wilcoxon test of 3-d averaged values between the two diet arms. **e**, Variance explained by diet ($n=156$ samples across 10 subjects) as quantified by R^2 values using Adonis tests for each subject (*$P<0.05$). **f**, Shannon diversity over time, labeled and analyzed as in panel **d** ($n=156$ samples). **g**, Relative abundance of sequence variants that were significantly different between diet arms (FDR-adjusted *P* value, $Q<0.1$, DESeq2 with two-sided Wald test) over time. Lines represent mean ± s.e.m. **h**, Relative abundances of bacterial species based on metagenomics that were significantly different between diet arms ($n=34$ samples from 17 individuals; FDR < 0.05, paired Wilcoxon test). Each point represents a single sample. Letters in panels **b**, **c** and **e** indicate subject identity. Diet order in panels **a**, **g** and **h** is indicated by color: red, OF first; gray, UF first. *P* values in panels **b** and **c** from Adonis tests. PCo1, principal coordinate 1.

Our previous study[5], designed to compare stool calorie loss in lean versus obese participants given diets of identical calorie content, found that UF decreased the ratio of Firmicutes to Bacteroidetes and increased relative stool calorie loss. In lean individuals, the decreased Firmicutes to Bacteroidetes ratio was associated with greater relative stool calorie loss, indicating decreased



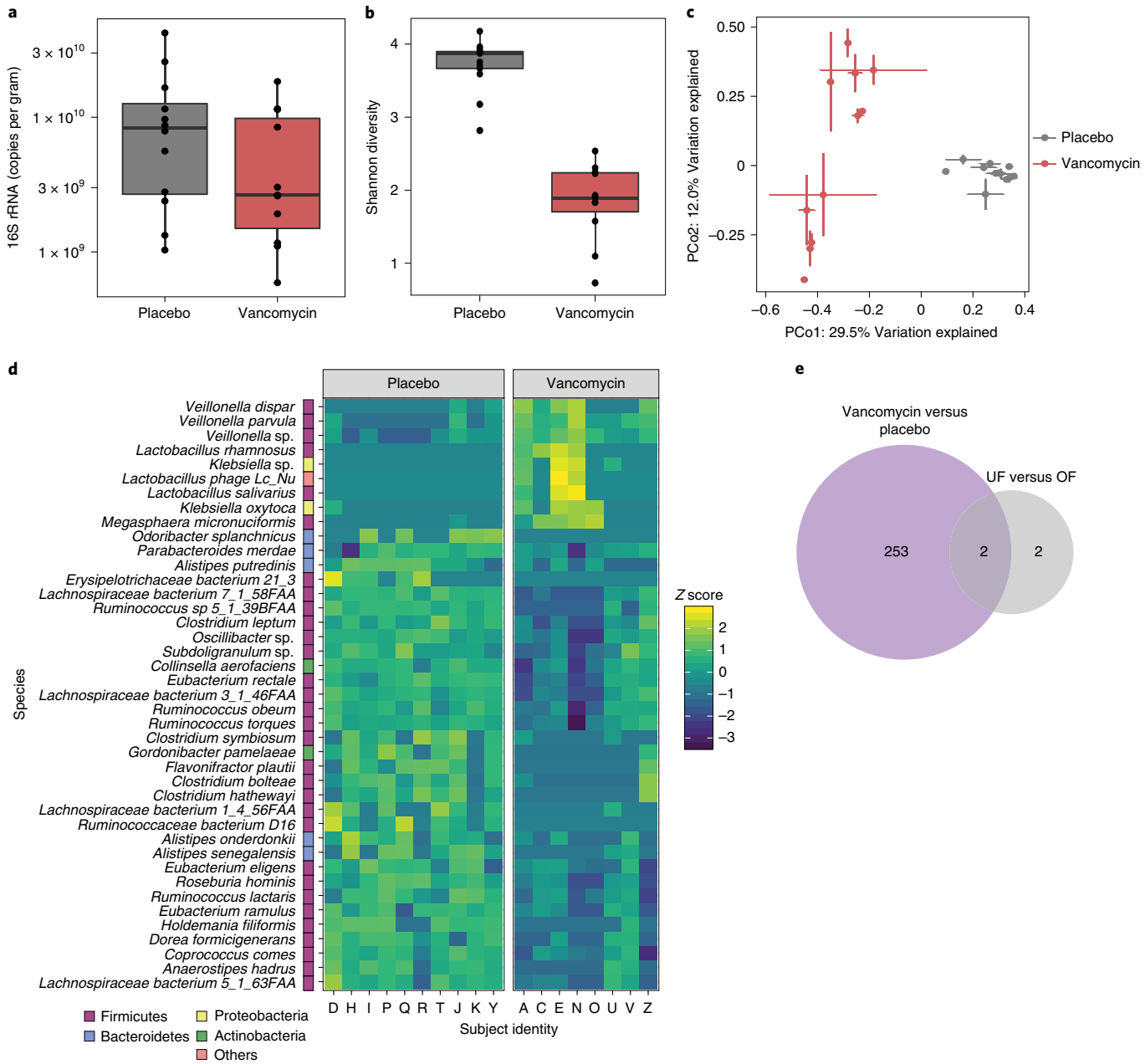


Fig. 5 | Changes in the gut microbiome in response to oral vancomycin. a, Overall gut microbial colonization, represented as 16S rRNA gene copies per gram wet weight, between vancomycin and placebo groups ($P = 0.26$, two-sided unpaired Wilcoxon test). Each data point represents the mean value of samples from each subject ($n = 63$ samples from 23 subjects). **b**, Shannon diversity in microbial communities of vancomycin and placebo groups ($P < 0.0001$, two-sided unpaired Wilcoxon test). Each data point represents the mean value of samples from each subject ($n = 70$ samples from 24 subjects). Boxplots in **a** and **b** indicate the interquartile range (IQR, 25th to 75th percentiles), with a center line indicating the median and whiskers showing the value ranges up to 1.5 × IQR above the 75th or below the 25th percentiles. **c**, Principal coordinate analysis of Bray–Curtis dissimilarity for ordination of microbial communities of subjects on vancomycin or placebo. Each data point shows the mean and standard deviation of samples from each subject ($n = 70$ samples from 24 subjects; $P < 0.0001$, determined using Adonis comparing effect of treatment and accounting for repeated measures per subject). **d**, Heatmap of differentially abundant species, determined by shotgun metagenomic sequencing, between vancomycin ($n = 8$) and placebo ($n = 10$) groups (FDR < 0.05, two-sided unpaired Wilcoxon test). Letters represent subject identity. **e**, Venn diagram of 16S rRNA sequence variants significantly different between vancomycin and placebo, or UF and OF, respectively (FDR < 0.1, two-sided unpaired or paired Wilcoxon test). Two bacterial species consistently changed in response to both perturbations: *A. muciniphila* and an unidentified species belonging to *Lachnospiraceae* NK4A136 group.

nutrient absorption. Our current study differs by more carefully controlling OF and UF by systematically providing diets that were 50% or 150% of participants' weight-maintaining needs. We confirmed and quantified the effect of UF on increasing relative stool calorie loss. However, in contrast to our previous results, we did not observe significant changes at the phylum level. The difference in our current study may have been due to the more carefully controlled dietary conditions, with each participant receiving the exact same foods for each 3-d OF and UF period, indicating that factors other than calorie load (for example, differing meal content) may have led to our previously observed changes in phylum-level bacterial relative abundance.

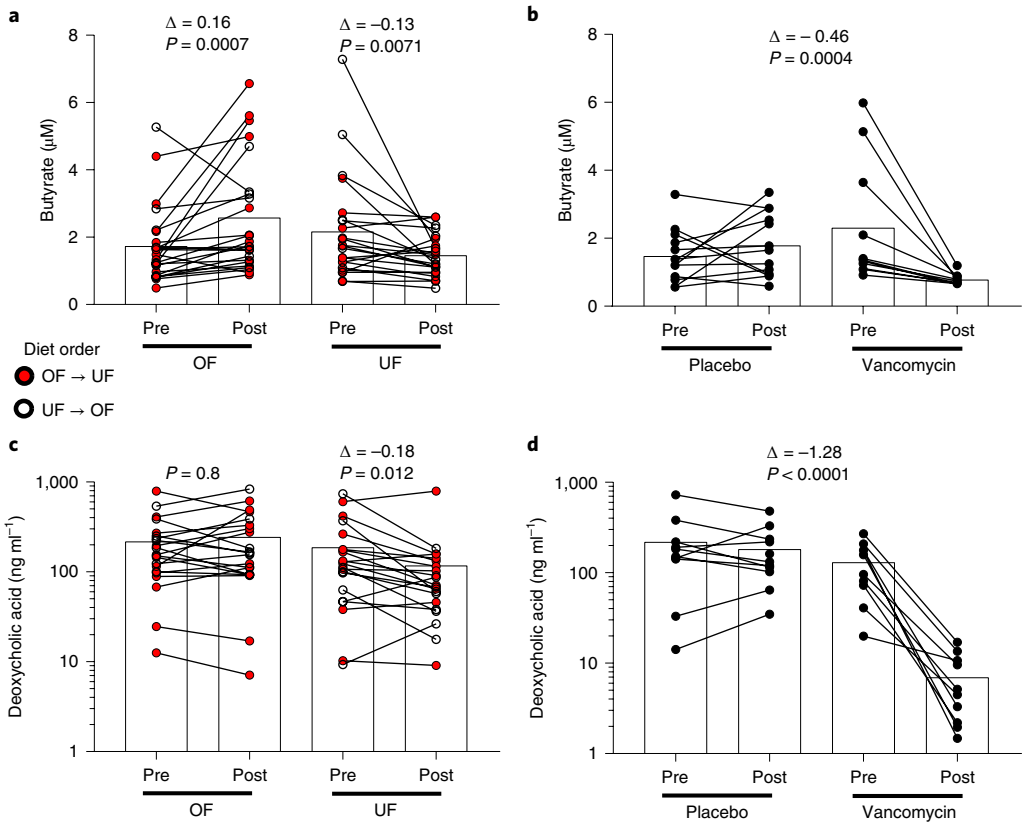


Fig. 6 | Common changes in plasma butyrate and deoxycholic acid following dietary and pharmacologic interventions. a,**b**, Mean butyrate concentrations during OF ($n=24$) and UF ($n=23$) (**a**) and during vancomycin ($n=11$) versus placebo ($n=13$) groups (**b**). Δ indicates the difference (log expressed) between pre- and postdietary intervention measurements (**a**) and the difference (log expressed) between pre- and postrandomization measurements in vancomycin and placebo groups (**b**). **c**,**d**, Mean deoxycholic acid concentrations (ng ml^{-1}) during OF ($n=21$) and UF ($n=21$) (**c**) and during vancomycin ($n=10$) versus placebo ($n=11$) groups (**d**). Δ indicates the difference (log expressed) between pre- and postdietary intervention measurements (**c**) and the difference (log expressed) between pre- and postrandomization measurements in vancomycin and placebo groups (**d**). *P* values determined by two-sided paired Student's *t*-test (**a**, **c**) or two-sided Student's unpaired *t*-test (**b**, **d**). Participants who started the dietary phase with OF are represented by red dots, while those who started with UF are represented by white dots. The sample size for each intervention refers to the volunteers who had available measurements of butyrate and/or deoxycholic acid concentrations.

Our results also differ from another carefully conducted study[17] that did not find an effect of oral vancomycin on stool calories despite widespread changes in gut microbial community structure and metabolite concentrations. We carefully controlled ingested calories, with both placebo and vancomycin groups receiving the exact same meals. By measuring ingested calories, we were able to calculate percentage of stool calories absorbed. This is important, as labeled calories reflect 'metabolizable' calories whose accuracy is problematic for careful energy balance studies such as these[38–41]. Nevertheless, we found differences in both absolute and percentage stool calories. The difference in actual calories of ~80 kcal d^{-1} appears to be comparable to the difference in stool calories between placebo and vancomycin groups in the study by Reijnders et al.[17].

Our results provide insights into potential mechanisms that could explain the increased stool calories loss. Both perturbations (UF and vancomycin) resulted in a significant increase in the relative abundance of *A. muciniphila*, which is recognized for its ability to forage on host mucins[42]. In mice, *A. muciniphila* counteracts the effect of the decrease in mucous layer thickness produced by a high-fat diet[26], producing weight loss without changes in food intake. *A. muciniphila* increases turnover of the mucous layer preventing disruption of tight junctions, which may reduce gut permeability[43,44]. In humans, a randomized, double-blind, placebo-controlled pilot study revealed a trend towards decreased body weight and fat mass in response to *A. muciniphila* supplementation[45]. Administration of pasteurized *A. muciniphila* cells[45] in this study increased stool energy content, consistent with our results. Additional studies are warranted to elucidate the role and mechanisms of *A. muciniphila* in nutrient absorption.

Both perturbations led to a significant decrease in plasma butyrate and deoxycholic acid concentrations, consistent with earlier studies[17]. These two compounds play opposing roles in the gastrointestinal tract, affecting gut barrier function and modifying epithelial layer nutrient permeability. In vitro, deoxycholic acid reduces both the tight junction protein zonula occludens-1 and mucin-producing goblet cells and increases intestinal macrophage polarization, suggesting that deoxycholic acid interferes with intestinal barrier function and induces intestinal inflammation[34]. Butyrate, on the other hand, has a salutary effect on gut barrier function[46]. In vitro studies show that butyrate and other short-chain fatty acids stimulate intestinal barrier formation, inducing protection against disruption from LPSs[47]. Our observations suggest that caloric intake and antibiotics may disrupt the balance between these, and likely other, microbial metabolites that regulate barrier function, resulting in altered nutrient absorption.

We evaluated alternative mechanisms that could explain the observed differences in nutrient absorption. We did not find evidence that circulating markers of gut permeability (plasma LPS[36] and zonulin[35,48,49]) decreased with interventions that led to reduced

nutrient absorption. Energy content in stool may also be affected by colonic transit time, which was more rapid with UF. In rats, a reduced transit time increases fecal volume and suppresses fat absorption[50]. During isocaloric diets of similar macronutrient but differing fiber content, high-fiber (expected to increase stool calorie loss) decreases transit time compared with low-fiber diets[51]. For UF, more rapid transit could have decreased contact time with gut epithelium, decreasing absorption.

We do acknowledge several limitations. Our sample size was small, yet in this carefully controlled inpatient study we had a diverse participant population. The study was powered to detect differences in stool calorie excretion between UF and OF and vancomycin versus placebo. Thus, we lacked power to detect associations between the gut microbiota and stool calorie loss. Also, we acknowledge that a direct causal role for the bacterial species, genes and metabolites associated with energy harvest needs to be tested in animal models and controlled human intervention studies.

The UF and OF periods were only 3 d, and it is possible that more dramatic shifts in the gut microbiota might occur with more prolonged dietary interventions. Oral vancomycin was the only antibiotic tested, so we do not know whether other antibiotics might have an effect on stool calories using our study design. We also did not have stool samples or dietary records before admission to determine how different our study diets were to free-living diets. Any proposed mechanisms regarding nutrient absorption were inferred as we are not able to directly assess nutrient transit across the intestine.

The functional role of the human gut microbiome in nutrient absorption remains a topic of intense interest in the field of energy balance. In our study, both calorie restriction and oral vancomycin led to increased stool calorie loss and lower levels of plasma butyrate. The magnitude of this effect is ~2.5% of ingested calories, which would translate to a ~1.2-kg weight loss in a 100-kg subject over 1 yr (ref. [52]). By limiting nutrient availability to the microbiome by calorie restriction or altering the ability of the microbiome to metabolize calories by widespread change in community structure, our data provide evidence for a causal role for the human gut microbiome in nutrient metabolism.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-020-0801-z.

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Methods

Study volunteers. From March of 2014 to May of 2017, 51 individuals aged 18–45 yr were recruited from the greater Phoenix area. Of these, 27 met initial inclusion criteria and were admitted to our CRU. Participants were fully informed of the study and signed consent forms before any study procedures. Except for impaired glucose tolerance and obesity, all participants were deemed healthy by medical history, physical exam and laboratory testing (see inclusion criteria and exclusion criteria below). The experimental protocol was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases.

Inclusion criteria. Inclusion criteria were as follows: free of acute and chronic diseases (especially gastrointestinal disorders) as determined by medical history, physical examination and laboratory tests; no laxative drugs within 3 weeks of admission; and age 18–45 yr (to minimize the effect of aging on nutrient absorption).

Exclusion criteria. Specific exclusion criteria included use of antibiotics or probiotics within 3 months and history of lactose intolerance. During the initial part of the inpatient admission, participants were tested for stool occult blood, stool ova and parasites, and celiac disease (total immunoglobulin A and anti-tissue transglutaminase antibodies). We excluded volunteers with chronic diseases or current substance abuse and subjects with a history or clinical manifestation of: current smoking; type 2 diabetes (according to the World Health Organization diagnostic criteria); endocrine disorders, such as Cushing's disease, pituitary disorders, and hypo- and hyperthyroidism; HIV infection (self-report), due to effects on weight and body composition of HIV and medications used to treat HIV; active tuberculosis (self-report); asthma on active daily treatment with medications; pulmonary disorders, including physician-diagnosed chronic obstructive pulmonary diseases and obstructive sleep apnea syndrome; cardiovascular diseases, including coronary heart disease, heart failure, arrhythmias and peripheral artery disease; hypertension (according to the World Health Organization diagnostic criteria), treated or uncontrolled; and gastrointestinal diseases, including inflammatory bowel diseases (for example, Crohn's disease and ulcerative colitis), malabsorption syndromes (for example, celiac disease), gastric ulcer (active) and irritable bowel syndrome. All participants had negative testing during admission for anti-tissue transglutaminase antibodies and stool blood. Additional exclusions included: lactose intolerance by medical history; anemia (defined as hemoglobin $< 11\,mg\,dl^{-1}$), leucopenia (defined as white blood cell count $< 4{,}000\,\mu l^{-1}$) or thrombocytopenia (defined as platelet count $< 150{,}000\,\mu l^{-1}$); liver disease, including nonalcoholic fatty liver disease or current elevated liver enzymes over 1.5× the normal range for aspartate aminotransferase (AST), alanine aminotransferase (ALT) or gamma-glutamyl transferase (GGT); evidence of chronic renal disease as defined by estimated glomerular filtration rate of $<60\,ml\,min^{-1}$ or evidence of overt proteinuria on urine dipstick; central nervous system disease, including previous history of cerebrovascular accidents, dementia and neurodegenerative disorders; cancer requiring treatment in the past 5 yr, except for nonmelanoma skin cancers or cancers that have clearly been cured or in the opinion of the investigator carry an excellent prognosis; behavioral or psychiatric conditions that would be incompatible with a safe and successful participation in the study (such as major depression, schizophrenia and presence of psychotic symptoms); eating disorders such as anorexia nervosa, bulimia or binge eating syndrome; taking weight-loss drugs; weight change of more than 5% of total body weight in the 3 months before admission; use of any antibiotic or probiotic agents within 6 months before the study, to minimize the potential effects of these substances on the gut microbiota; use of antacids (proton pump inhibitors, H_2 antagonists or aluminum/magnesium hydroxide) 3 months before the study assessed by self-report; and evidence of alcohol and/or drug abuse (more than three drinks per day and use of drugs, such as amphetamines, cocaine, heroin or marijuana). Finally, we excluded subjects with known allergies to vancomycin, known allergies to heparin or a history of heparin-induced thrombocytopenia, or a personal history or evidence of a bleeding disorder.

Study design and randomization. This was a controlled dietary study conducted in two contiguous phases. Numbered randomization lists for diet and pharmacologic interventions by sex were generated before start of study by statistician and participants were assigned by enrollment (for example, Male no. 1, Male no. 2, Female no. 1, Female no. 2 and so on). The first phase was a cross-over design in which all participants underwent in random order 3 d of OF and UF, followed by a second phase involving double-blind, randomized, placebo-controlled assignment to administration of oral vancomycin or matching placebo. Individuals were admitted to the CRU for approximately 31 d (Fig. 1). Throughout the study, subjects were fed a WMD (20%, 30% and 50% of daily calories were provided as protein, fat and carbohydrate, respectively). This was based on unit-specific equations specific to our CRU that include weight, BMI and sex[53]. Morning weight was checked daily, and the calories were adjusted as necessary throughout the stay to maintain a stable weight (±1%). Participants were required to consume all provided food during the study. Total-body dual-energy X-ray absorptiometry (Lunar Prodigy, enCORE 2003 software version 7.53.002, GE Lunar) to assess body composition was performed on day 2. A 75-g oral glucose tolerance test (OGTT) was done on day 4 to identify individuals with diabetes[54]. Starting on day 5, participants were fed in random order (permuted block randomization by sex with a block size ranging from 1 to 5) 50% (UF) and 150% (OF) of their WMD each separated by 3 d of WMD. For the UF and OF diets, the same exact menus (same foods) were given in the same order over the 3-d period, only varying in calorie content (Supplementary Table 12). Fasting blood samples were collected before and following the UF and OF diets. Urine was collected during each 3-d period. To mark stool related to each diet, a tasteless, nonabsorbable, blue dye (FD&C 250 mg) was given to the participant just before breakfast on day 1 of each UF and OF diet and just before breakfast on the day following each OF and UF diet. Stool for calorie measurement was collected from appearance of the first dye marker (dyed stool included) until appearance of the second one (dyed stool excluded). Following the UF and OF phase, participants were placed back on WMD. After 3 d back on WMD, they underwent an OGTT, followed the next day by measurement of 24 h energy expenditure and substrate use in a whole-room indirect calorimeter.

After exiting the calorimeter and while fasting, an intravenous bolus of $75\,IU\,kg^{-1}$ heparin was administered and, 15 min after the bolus, blood was collected for measurement of LPL and ANGPTL4. Volunteers were then randomly assigned to receive either vancomycin 125 mg orally four times a day or identical placebo pills for 14 d. The randomization and release of the medication were managed by the pharmacy at the Phoenix Indian Medical Center using a permuted block randomization by sex, with a block size ranging from 1 to 5. The experimental design is shown in Fig. 1. Fasting blood samples were obtained on randomization day (day 18) and following the treatment period (day 31). After 5 d on vancomycin or placebo, stool calorie measurements were again collected over 3 d while patients continued WMD. Following this, the OGTT, 24-h energy expenditure, LPL and ANGPTL4 measures were repeated.

Calorie measurement of food, urine and stool by bomb calorimetry. Volunteers ate under supervision and consumed at least 95% of the provided food. During each 3-d period of determination of stool, urine and food calories, meals were prepared in duplicate and one given at random to the participant. The duplicate meal was then used for direct determination of calories. Calories in food, urine and stool were measured as previously described[5]. Briefly, stool samples were collected from appearance of the first marker to appearance of the second marker and weighed. An aliquot of stool was then mixed with distilled water equal to weight of the fecal aliquot. This was then homogenized and lyophilized[55]. Similarly, food samples were made into a slurry and were lyophilized. Urine samples were directly lyophilized. Lyophilization was performed at −77 °C using a Freezemobile12XL instrument (VirTis). Following this, ~1-g pellets were made from (PARR Instrument Co) dried stool, food and urine. Energy content of pellets was measured using bomb calorimetry using the Isoperibol Calorimeter 6200 instrument with a 1108 Oxygen Bomb model (PARR Instrument Co). Samples were run in duplicate and results averaged. If duplicate samples were >40 cal apart, calorimetry was repeated until duplicate samples were <40 cal apart or the mean of four consecutive readings was used. Benzoic acid standards were measured every 20 burns for standardization. The calorie loss from stool or urine was calculated as kilocalories per day and as percentage calories of ingested food ((stool calories/ingested calories) × 100)). Transit time was calculated as the difference between the appearance of the first dye marker and the appearance of the second dye marker during each period of stool collection.

Energy expenditure and macronutrient oxidation. Twenty-four-hour energy expenditure was measured by whole-room indirect calorimetry as previously described[56]. Briefly, participants entered the chamber after breakfast then stayed inside for ~23.5 h. The air temperature was maintained at 23 °C. Meals were provided through an airtight interlock at 11:00, 16:00 and 19:00. Spontaneous physical activity was continuously monitored by a radar system based on the Doppler effect. The flow rate through the chamber and the CO_2 and O_2 concentrations of the out-flowing air were continuously monitored and calculations of oxygen consumption, carbon dioxide production and respiratory quotient were made every minute. Calculation of energy expenditure and substrate oxidation was performed using the equations of Lusk[57]. Sleeping energy expenditure was determined between 23:30 and 5:00 when spontaneous physical activity (SPA) was <1.5%. All measures were extrapolated to 24 h.

Biochemical analyses for plasma variables. Leptin was measured using the human leptin Quantikine ELISA kit from R&D Systems. The inter- and intra-assay coefficient of variation (%CV) values were 1.58 and 1.34, respectively. Active GLP-1 was measured using the Version2 Multi Array Assay kit from Mesoscale Discovery. The inter-assay and intra-assay %CV values were 3.99 and 2.89, respectively. GLP-2 was measured using the GLP-2 ELISA kit from EMD Millipore. The inter- and intra-assay %CV values were 3.33 and 2.80, respectively. Plasma ANGPLT4 was measured using the sandwich ELISA from BioVendor. The intra-assay %CV was 1.58 and the inter-assay %CV was 4.46. The LPL activity was measured using the assay kit from ROAR Biomedical. The intra-assay %CV was 1.83. Plasma short-chain fatty acids (SCFAs) were performed in duplicate

on a Waters Acquity UPLC-Thermo QExactive high-resolution/accurate mass spectrometer. Plasma primary and secondary bile acid concentrations were measured using liquid chromatography–mass spectrometry (LC-MS) analysis performed in duplicate on a Thermo Vanquish UPLC-Thermo Altis triple-quadrupole mass spectrometer. Plasma glucose was measured by the glucose oxidase method (Beckman Instruments or GM9 Glucose Analyzer, Analox Instruments). Fasting insulin concentrations were assessed using an automated immunoenzymometric assay (Tosoh Bioscience). LPS and zonulin were measured using an assay from CusaBio Technologies. The LPS intra-assay %CV was 6.77 and the inter-assay %CV was 9.9. The zonulin intra-assay %CV was 5.46 and the inter-assay %CV was 5.87.

Power calculation. Power was based on two primary outcomes, the difference in stool calories between UF and OF and the difference in stool calories between vancomycin and placebo. Based on our previous studies[5], 24 individuals gave us a power of 0.86 at an alpha of 0.05 to detect at least a 1.3 ± 1.9% per day (mean ± s.d.) difference in stool calories between UF and OF. For the vancomycin versus placebo, 12 subjects per group gave us a power of 0.95 at an alpha of 0.05 to detect a 75 ± 49 kcal d^{-1} (mean ± s.d.) difference in stool calories.

Quantification of bacterial load. DNA templates were diluted 100-fold before quantitative PCR (qPCR) of 16S rRNA gene copies was performed. qPCR was carried out in triplicate 10-µl reactions with 200 nM 340 F (forward)/514 R (reverse) primers using a BioRad CFX384 thermocycler with SYBRSelect for CFX Master Mix (Life Technologies) according to the manufacturer's instructions and an annealing temperature of 60 °C. Absolute quantifications were determined against a standard curve of purified bacterial DNA. Mean values of triplicate reactions were taken for further downstream analyses. Absolute bacterial abundance was derived by adjustments for dilutions during DNA extraction, normalization and PCR reaction preparation, dividing by the total fecal mass used for DNA extraction in grams.

16S rRNA gene sequencing and analysis. Stool samples were collected daily and immediately frozen at −70 °C until processing. Stool samples were homogenized with bead beating for 5 min (Mini-Beadbeater-96, BioSpec) using the ZR BashingBead lysis matrix containing 0.1- and 0.5-mm beads (ZR-96 BashingBead Lysis Rack, Zymo Research) and the lysis solution provided in the ZymoBIOMICS 96 MagBead DNA Kit (Zymo Research). The samples were then centrifuged for 5 min at 3,000*g* and the supernatant was transferred to 1-ml deep-well plates. The DNA was then purified using the ZymoBIOMICS 96 MagBead DNA Kit (Zymo Research) according to the manufacturer's instructions. Using a previously published protocol and primers, 16S rRNA gene PCR was carried out [58]. Amplicons were pooled and normalized using the SequalPrep Normalization Plate Kit (Invitrogen). The normalized amplicon pool was concentrated down to ~100 µl in a SpeedVac and then purified using 1× AMPure XP beads (Beckman Coulter). Libraries were quantified using the KAPA Library Quantification Kit (Illumina) and sequenced using 2 × 250-base pair paired-end sequencing (Illumina MiSeq) with 15% PhiX spike-in. Demultiplexed reads were processed using QIIME2 (ref. [59]) with denoising by DADA2 (ref. [60]). Taxonomy was assigned using the DADA2 implementation of the RDP classifier[61] using the DADA2-formatted training sets for SILVA128 (benjjneb.github.io/dada2/assign.html). A phylogenetic tree was constructed via fragment insertion in QIIME2 (qiime fragment-insertion sepp). Sequence variants were filtered such that they were present in more than one sample with a total of at least 10 reads and a relative abundance of at least 0.0001 (0.01%) across the dataset. Samples with lower total read count than template control were discarded. Diversity metrics were generated using Vegan[62] with principal coordinate analysis carried out with APE[63]. Differential abundance analysis of sequence variants was carried out using DESeq2 (ref. [64]). Significant sequence variants (FDR < 0.05) were visualized on a phylogenetic tree using ggtree[65].

Metagenomic sequencing and analysis. For a subset of samples that were subjected to 16S rRNA amplicon sequencing, the same DNA extracts were subjected to shotgun metagenomic sequencing. Whole-genome shotgun libraries were prepared using the Nextera DNA Flex Library Preparation Kit (Illumina) with the Nextera DNA Unique Dual Indexes and sequenced on the NovaSeq S2 platform as 2 × 100-base pair paired-end reads. Raw Illumina reads underwent quality trimming and adaptor removal using Fastp[66] and host read removal by mapping to the human genome (GRCh38) with Bowtie2 (ref. [67]). Metagenomic samples were taxonomically profiled using MetaPhlan2 (ref. [68]) and functionally profiled using HUMAnN2 v0.11.1 (ref. [69]), both with default parameters. Species from taxonomic profiles were retained for further analysis if their mean relative abundance exceeded 0.0001 (0.01%) across the dataset with nonzero abundances in at least two samples. Tables of pathway and gene family abundance obtained using HUMAnN2 were normalized to copies per million using 'humann2_renorm_table -units cpm', including unmapped and unintegrated read mass. Pathway abundance files were joined into one abundance table and filtered to contain the pathways with top 75% mean abundance (final $n = 290$ pathways). Gene family files were regrouped using the humann2_regroup_table command with the 'uniref90_ko' term to KEGG Orthology groups of functional orthologs[70], thereby reducing the number of low-abundance variables and number of tests to be performed downstream. Regrouped, per-sample KEGG Orthology abundance profiles were joined into one abundance table and filtered to contain features with top 75% mean abundance (final $n = 3,553$ KEGG Orthology groups). Differential abundance analyses of pathways and KEGG Orthology groups were carried out using Wilcoxon test with multiple testing correction via FDR estimation across features tested.

Statistical analysis. Statistical analyses were performed using the procedures of SAS, v.9.2 (SAS Institute). Normally distributed data are presented as mean ± s.d.; data that deviated from normality are presented as median and 95% confidence interval. To fit linear regression models, skewed variables were $\log_{10}$ transformed to approach a normal distribution assessed by tests for normality. Pearson or Spearman (for non-normally distributed variables) correlation was used to test associations between variables. In OF versus UF, changes in stool and urine calories expressed as total and percentage of ingested calories, hormones and short-chain fatty acids were compared by paired Student's *t*-test. In the vancomycin versus placebo phase, the same variables were compared using an unpaired Student's *t*-test. Changes in energy expenditure and substrate oxidation before and after vancomycin and placebo were evaluated by analysis of covariance accounting for baseline values. For energy expenditure and substrate oxidation measures, models included adjustment for baseline fat-free mass (FFM), fat mass (FM), age, race and sex. Mixed models using a first-order autoregressive covariance structure were used to analyze weight trajectory during the inpatient stay. Glucose area under the curve and insulin area under the curve were calculated using the trapezoidal method. Paired and unpaired Wilcoxon tests were used to compare changes in the relative abundance of taxonomic (phyla, species) and functional (pathways, KEGG Orthology groups) features for UF versus OF and vancomycin versus placebo, respectively. Bacterial load was calculated as number of 16S rRNA gene copies per gram of feces. Shannon diversity index was used to determine the microbial diversity between interventions. Bray–Curtis dissimilarity was used to quantify compositional dissimilarity between microbial communities and the distance matrix was analyzed using Adonis PERMANOVA (R Vegan package) with 10,000 replications.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data in the published article and its Supplementary Information have been presented where possible in aggregated form. The individual datasets generated during and/or analyzed during the current study are available from the corresponding author (A.B.) upon request, although restrictions may apply due to patient privacy and the General Data Protection Regulation. All sequencing data generated in the preparation of this manuscript have been deposited in NCBI's Sequence Read Archive, with accession number PRJNA589622. Source data for Figs. 2–6 and Extended Data Figs. 2–8 are presented with the paper.

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Author contributions

J.K. and R.J.v.S. designed the study. A.B., M.H., S.H., P.P. and J.K. analyzed and interpreted the clinical data. M.W. and P.W. analyzed blood samples. S.P. assisted with laboratory procedures. Q.Y.A. and D.D.T. generated the microbiome data. Q.Y.A. led the data analysis. A.B. wrote the initial draft of the manuscript. J.K. and P.J.T. edited the submitted version. A.B., J.K. and P.J.T. are the guarantors of this work and have full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors critically revised the draft and approved the final manuscript.

Competing interests

P.J.T. is on the scientific advisory board for Kaleido, Pendulum, Seres and SNIPRbiome; there is no direct overlap between the current study and these consulting duties.

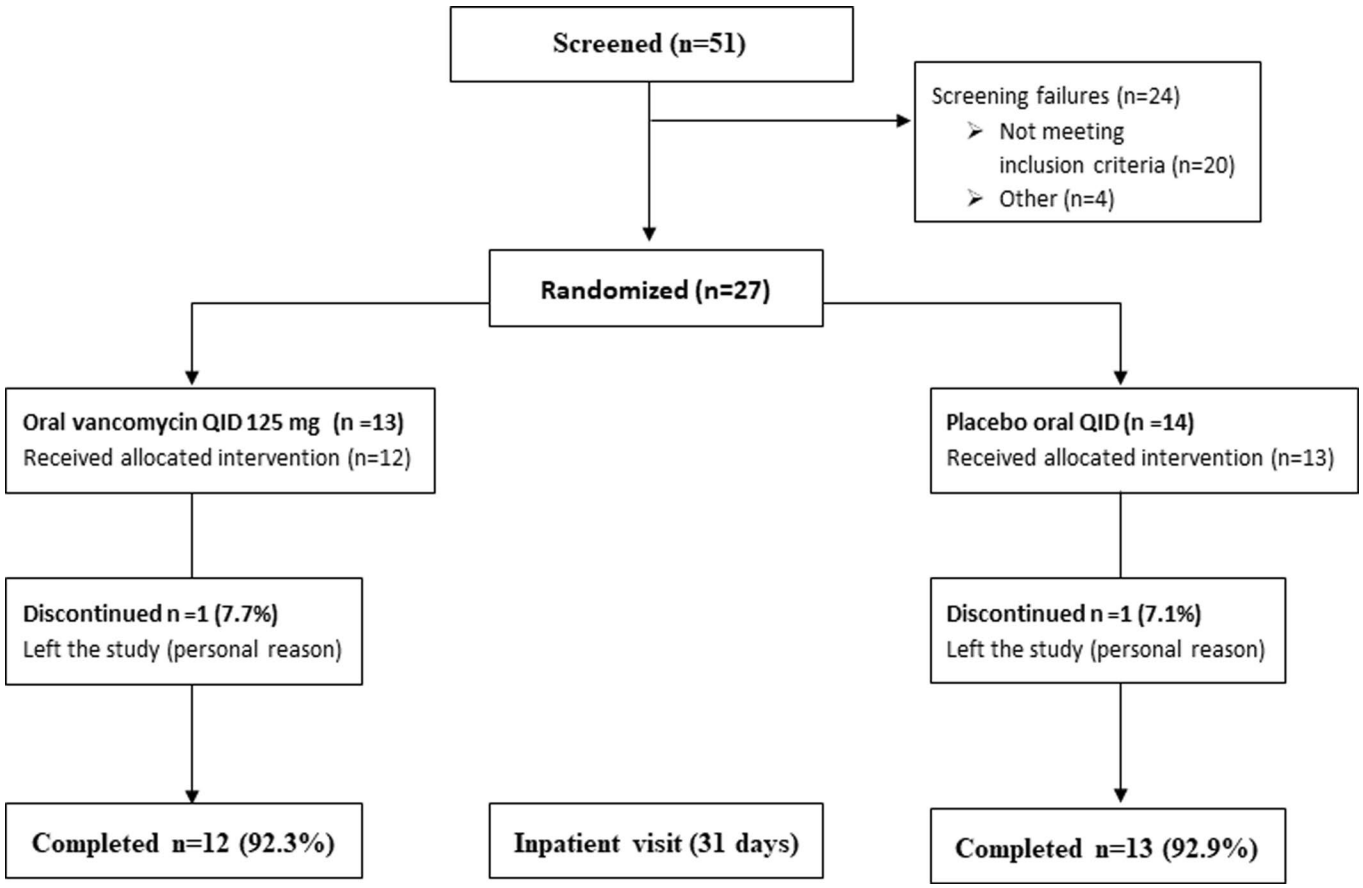
Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-020-0801-z.

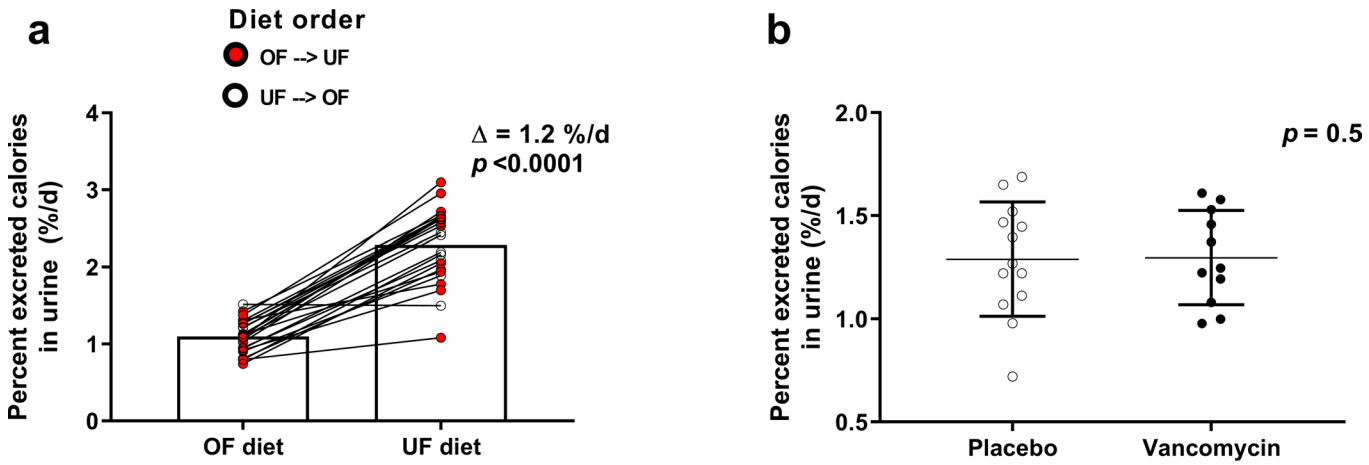
Supplementary information is available for this paper at https://doi.org/10.1038/s41591-020-0801-z.

Correspondence and requests for materials should be addressed to A.B., P.J.T. or J.K.

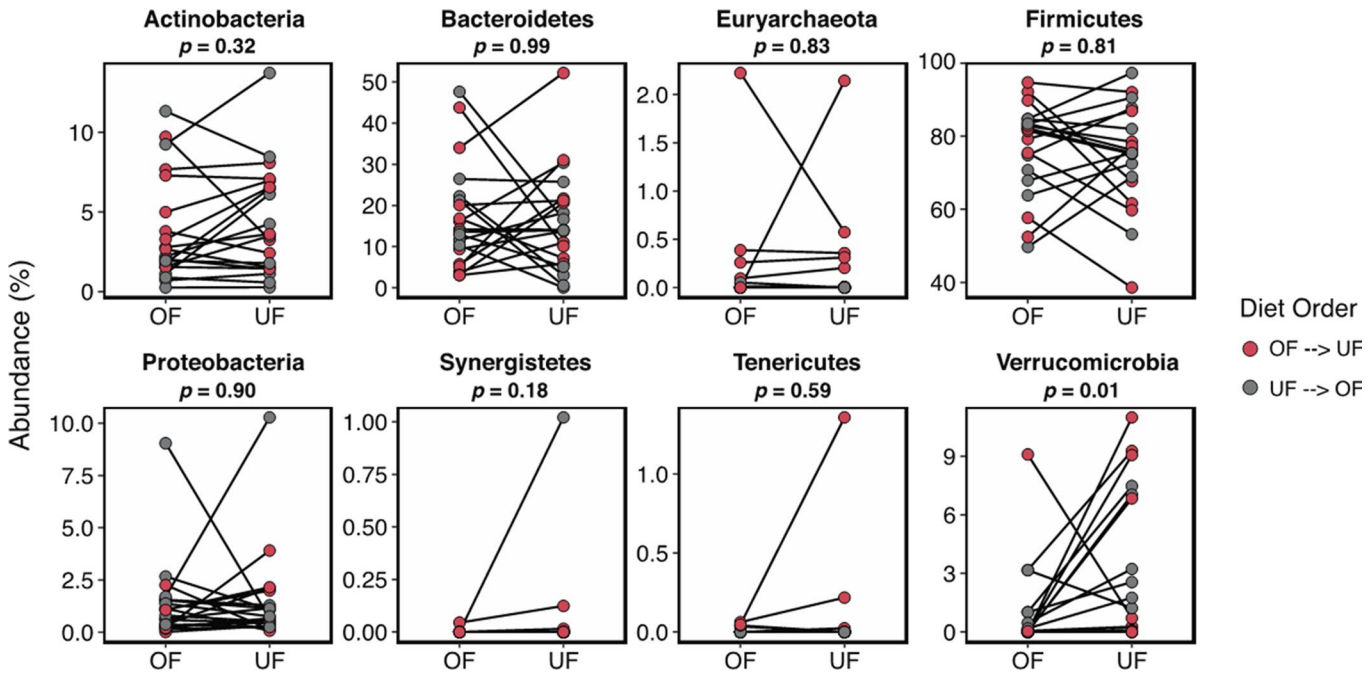
Peer review information Joao Monteiro was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.



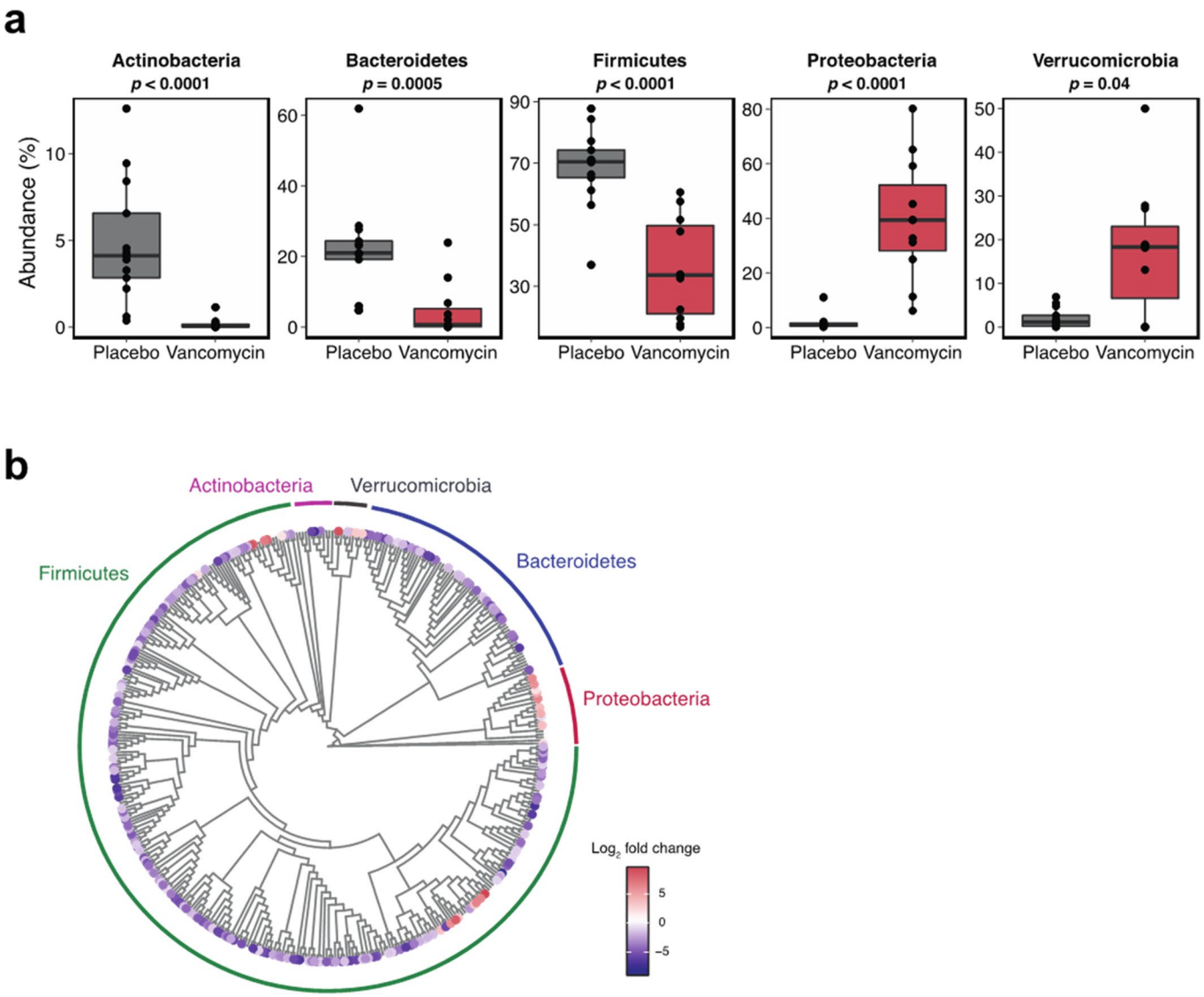
Extended Data Fig. 1 | Consort diagram for study protocol. Two individuals left the study due to personal reasons after completing the over and underfeeding portion of the study and after randomization to vancomycin (125 mg QID, 4 times per day) or placebo.



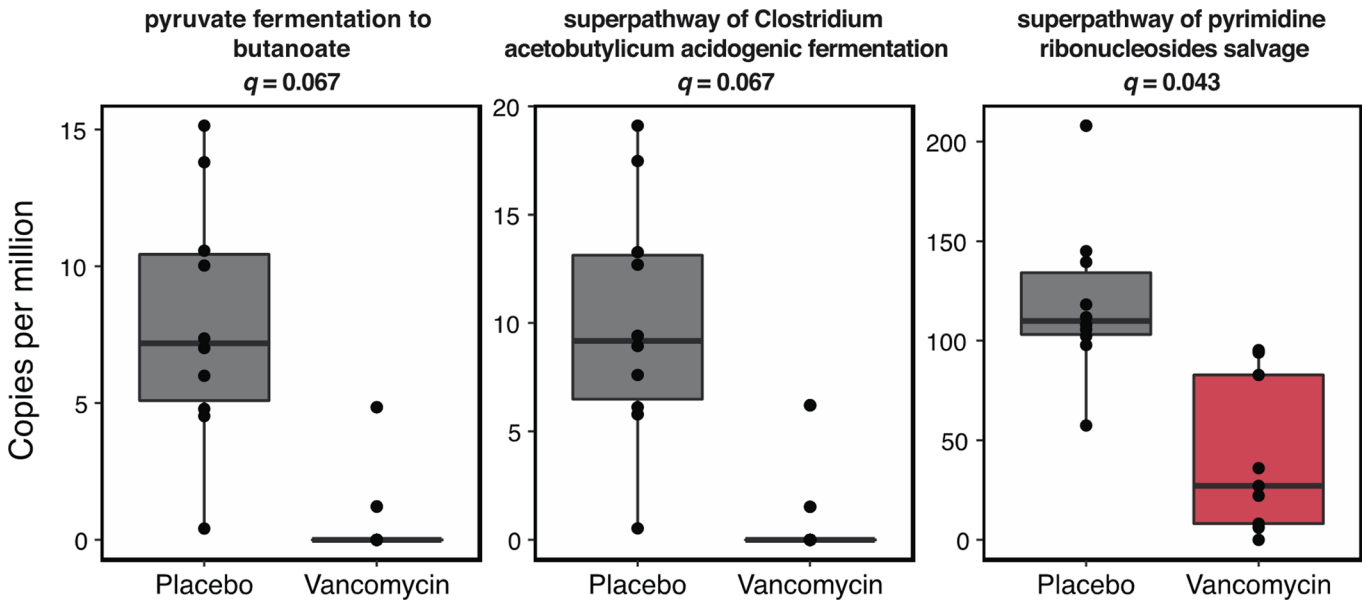
Extended Data Fig. 2 | Urine calorie content in response to dietary and pharmaceutical interventions. a, mean percent calorie loss in urine during overfeeding (n=25) and underfeeding (n=25). The Δ indicate the difference between underfeeding and overfeeding. Participants who started the dietary phase with overfeeding are represented by red dots, while those who started with underfeeding are represented by white dots. **b**, mean percent calorie loss in urine between vancomycin (n=11) and placebo (n=13) group. The sample size refers to the volunteers who had completed data of calorie loss from urine. *p*-values determined by two-sided paired (**a**) or two-sided unpaired Student's *t* test (**b**).



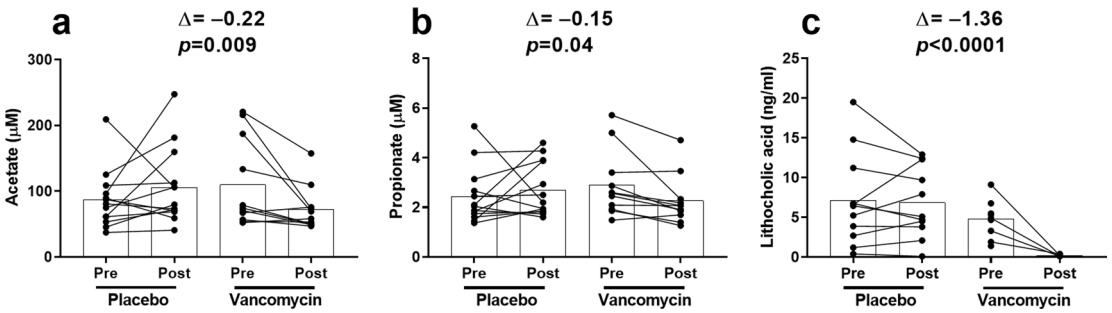
Extended Data Fig. 3 | Phylum-level relative abundances between overfeeding (OF) and underfeeding (UF). Data points represent single samples (n=40 samples) and are colored by randomization group for the dietary phase where participants who started the dietary phase with overfeeding are represented by red dots (n=11), while those who started with underfeeding are represented by grey dots (n=9). Samples from the same individual are connected by a line. *p*-values were determined by two-sided paired Wilcoxon tests.



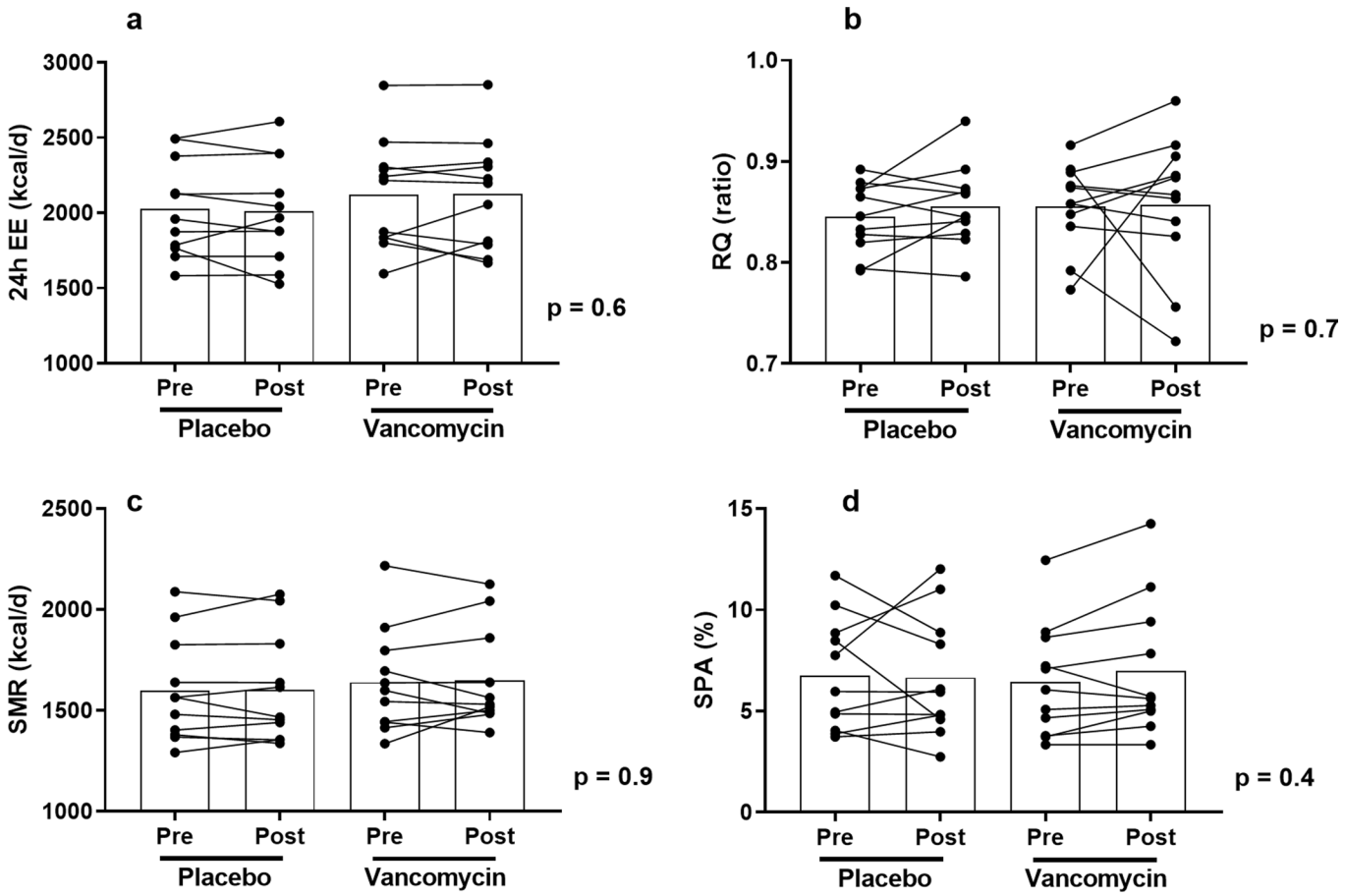
Extended Data Fig. 4 | Effect of vancomycin treatment on gut microbiota composition. Relative abundances of phyla significantly different between vancomycin and placebo groups (p<0.05, two-sided unpaired Wilcoxon test). Points indicate the mean value of samples from each subject (n=70 samples from 24 subjects). Boxplots indicate the inter-quartile range (IQR, 25th to 75th percentiles), with a center line indicating the median and whiskers showing the value ranges up to 1.5 x IQR above the 75th or below the 25th percentiles. **b**, Phylogenetic tree of 16S rRNA sequence variants (SVs) where colored tips denote significantly different SVs between vancomycin (n=11) and placebo (n=13) groups (FDR<0.1, DESeq2 with two-sided Wald test) with color representing fold change comparing vancomycin to placebo. Phylum distribution of the SVs are indicated around the tree.



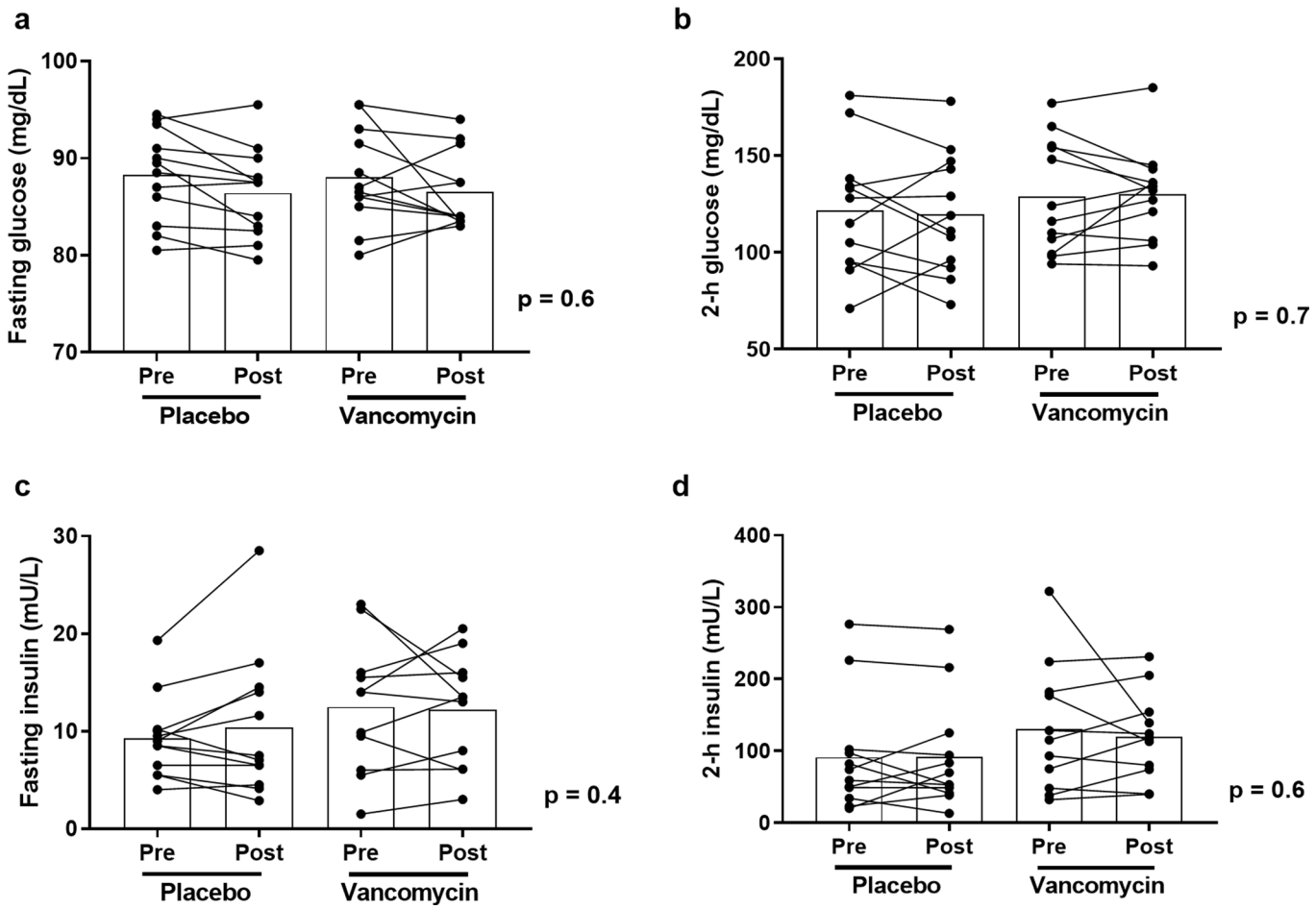
Extended Data Fig. 5 | Differentially abundant metabolic pathways between vancomycin and placebo. Three metabolic pathways are differentially abundant between vancomycin (n=9) and placebo (n=10) groups (FDR-adjusted *p*-value, $q<0.1$, two-sided unpaired Wilcoxon test). Each point represents a single sample. Boxplots indicate the inter-quartile range (IQR, 25th to 75th percentiles), with a center line indicating the median and whiskers showing the value ranges up to 1.5 x IQR above the 75th or below the 25th percentiles.



Extended Data Fig. 6 | Plasma acetate, propionate and lithocholic acid concentrations prior to and following treatment with oral vancomycin or placebo. a, mean acetate concentrations (μM) during vancomycin (n=11) versus placebo (n=13) group. **b**, mean propionate concentrations (μM) during vancomycin (n=11) versus placebo (n=13) group. **c**, mean lithocholic acid concentrations (ng/ml) during vancomycin (n=4) versus placebo (n=11) group. The values are expressed as absolute values. The Δ indicate the difference (log expressed) between pre and post randomization measurements in vancomycin and placebo groups. *p*-values for the difference in acetate, propionate and lithocholic acid concentrations before and after randomization by vancomycin vs. placebo group as determined by two-sided unpaired Student's *t* test. The sample size for each intervention refers to the volunteers who had available measurements of acetate, propionate and lithocholic acid concentrations.



Extended Data Fig. 7 | Twenty-four-hour energy expenditure (24h-EE) and respiratory quotient (RQ) pre and post-randomization in vancomycin and placebo groups. a, 24h-EE expressed as kcal/d; **b**, RQ, expressed as ratio; **c**, SMR expressed as kcal/d. **d**, SPA expressed as % of time spent moving over 24 hours. *p*-values indicate the change in 24h EE and its components (before and after randomization) between vancomycin (n=11) vs. placebo (n=11) groups as determined by two-sided Student's unpaired t-test. The sample size for each intervention refers to the volunteers who had available measurements of 24h EE and its components. 24h EE: 24 hours energy expenditure; RQ: respiratory quotient; SMR: sleeping metabolic rate; SPA: spontaneous physical activity.



Extended Data Fig. 8 | Glucose and insulin concentrations pre and post-randomization in vancomycin and placebo groups. a, mean fasting glucose (mg/dL); **b**, mean 2-h glucose concentration (mg/dL). **c**, mean fasting insulin concentration (mU/L). **d**, mean 2-h insulin concentration (mU/L). p values indicate the change in glucose and insulin concentration (fasting and at 120′ during the OGTT) before and after randomization between vancomycin (n=12) vs. placebo (n=12) groups as determined by two-sided Student's unpaired t-test. The sample size for each intervention refers to the volunteers who had available measurements of glucose and insulin during OGTT (oral glucose tolerance test). One volunteer in the vancomycin group did not have available measurement of fasting and 2-h insulin during OGTT.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

The first phase was a cross-over design in which all participants underwent in random order 3 days of over and underfeeding, followed by a second phase involving double blind randomized placebo-controlled assignment to administration of oral vancomycin or matching placebo. Participants were fed in random order 50% (UF) and 150% (OF) of their weight maintaining diet each separated by 3 days of WMD and volunteers were then randomly assigned to either receive vancomycin 125 mg orally four times a day or identical placebo pills for 14 days.

During each 3-day period of determination of stool. Stool samples were collected from appearance of first marker to appearance of the second marker and weighed during under- or overfeeding and after randomization to vancomycin.

Data analysis

Statistical analyses were performed using the procedures of SAS, version 9.2 (SAS Institute, Cary, NC).

Stool samples were collected from appearance of first marker to appearance of the second marker and weighed. An aliquot of stool was then mixed with distilled water equal to weight of the fecal aliquot. This was then homogenized and lyophilized 22. Similarly, food samples were made into a slurry and were lyophilized. Urine samples were directly lyophilized. Lyophilization was performed at −77°C using a Freezemobile12XL instrument (Virtis, Gardiner, NY). Following this, ~1 gram pellets were made from (PARR Instrument Co, Moline, IL) dried stool, food and urine.
Energy content of pellets was measured using bomb calorimetry using the Isoperibol Calorimeter 6200 instrument with a 1108 oxygen bomb model (PARR Instrument Co, Moline, IL). Samples were run in duplicate and results averaged. If duplicate samples were >40 calories apart, calorimetry was repeated until duplicate samples were <40 calories or the mean of 4 consecutive readings was used. Benzoic acid standards were measured every 20 burns for standardization.

Lunar Prodigy, enCORE 2003 software version 7.53.002, GE Lunar, Madison, WI, USA was used to assess total-body dual-energy X-ray absorptiometry (DXA) on day 2 of the study

Analysis of gut microbiota

16S rRNA Gene Sequencing and Analysis

Stool samples were collected daily and immediately frozen at -70°C until processed. Stool samples were homogenized with bead beating for 5 min (Mini-Beadbeater-96, BioSpec) using the ZR BashingBead lysis matrix containing 0.1 and 0.5 mm beads (ZR-96 BashingBead Lysis Rack, Zymo Research) and the lysis solution provided in the ZymoBIOMICS 96 MagBead DNA Kit (Zymo Research). The samples were then centrifuged for 5 min at 3,000 g and the supernatant was transferred to 1mL deep-well plates. The DNA was then purified using the ZymoBIOMICS 96 MagBead DNA Kit (Zymo Research) according to manufacturer's instructions. 16S rRNA gene PCR was carried out as per reference protocol and primers. Amplicons were pooled and normalized using the SequalPrep Normalization Plate Kit (Invitrogen). The normalized amplicon pool was concentrated down to ~100ul in a SpeedVac and then purified using 1x AmPure XP beads (Beckman Coulter). Libraries were quantified using the KAPA Library Quantification Kit (Illumina) and sequenced using 2x250 bp paired-end sequencing (Illumina MiSeq) with 15% PhiX spike-in. Demultiplexed reads were processed using QIIME2 with denoising by DADA2. Taxonomy was assigned using the DADA2 implementation of the RDP classifier using the DADA2 formatted training sets for SILVA128 (benjjneb.github.io/dada2/assign.html). A phylogenetic tree was constructed via fragment insertion in QIIME2 (qiime fragment-insertion sepp). Sequence variants were filtered such that they were present in more than one sample with at least a total of 10 reads and a relative abundance of at least 0.0001 (0.01%) across the dataset. Samples with lower total read count than template control were discarded. Diversity metrics were generated using Vegan with principal coordinate analysis (PCoA) carried out with Ape. Differential abundance analysis of sequence variants was carried out using DESeq2 . Significant sequence variants (FDR<0.05) were visualized on a phylogenetic tree using ggtree.

Quantification of Bacterial Load

DNA templates were diluted 100-fold before quantitative PCR (qPCR) of 16S rRNA gene copies was performed. qPCR was carried out in triplicate 10uL reactions with 200nM 340F/514R primers using a BioRad CFX384 thermocycler with SYBRSelect for CFX Master Mix (Life Technologies) according to the manufacturer's instructions and an annealing temperature of 60℃. Absolute quantifications were determined based against a standard curve of purified bacterial DNA. Mean values of triplicate reactions were taken for further downstream analyses. Absolute bacterial abundance was derived by adjustments for dilutions during DNA extraction, normalization, and PCR reaction preparation dividing by the total fecal mass used for DNA extraction in grams.

Metagenomic Sequencing and Analysis

For a subset of samples that were subjected to 16S rRNA amplicon sequencing, the same DNA extracts were subjected to shotgun metagenomic sequencing. Whole-genome shotgun libraries were prepared using the Nextera DNA Flex Library Preparation Kit (Illumina) with the Nextera DNA Unique Dual Indexes and sequenced on the NovaSeq S2 platform as 2 x 100 bp paired-end reads. Raw Illumina reads underwent quality trimming and adaptor removal using Fastp and host read removal by mapping to the human genome (GRCh38) with Bowtie2. Metagenomic samples were taxonomically profiled using MetaPhlan266 and functionally profiled using HUMAnN2 v0.11.1 both with default parameters. Species from taxonomic profiles were retained for further analysis if their mean relative abundance exceeded 0.0001 (0.01%) across the dataset with non-zero abundances in at least 2 samples. Tables of pathway and gene family abundance obtained using HUMAnN2 were normalized to relative abundance using 'humann2_renorm_table -units cpm' including unmapped and unintegrated read mass. Pathway abundance files were joined into one abundance table and filtered to contain the pathways with top 75% mean abundance (final n = 290 pathways). Gene family files were regrouped using the humann2_regroup_table command with the 'uniref90_ko' term to KEGG Orthology (KO) groups of functional orthologs, thereby reducing the number of low-abundance variables and numbers of tests to be performed downstream. Regrouped, per-sample KO abundance profiles were joined into one abundance table and filtered to contain features with top 75% mean abundance (final n = 3553 KOs). Differential abundance analysis of pathways and KO groups were carried out on normalized relative abundances and analyzed using Wilcoxon test with multiple testing correction via false discovery rate (FDR) estimation across features tested.

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All sequencing data generated in this study were deposited on the NCBI, under BioProject PRJNA589622. Other data supporting the findings of this study are available within the paper or available from the authors upon reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Power was based on two primary outcomes, the difference in stool calories between underfeeding and overfeeding and the difference in stool calories between vancomycin and placebo. Based on our previous studies, 24 individuals gave us a power of 0.86 at an alpha of 0.05 to

	detect a least a 1.3 ± 1.9 %/day (mean±SD) difference in stool calories between underfeeding and overfeeding. For the vancomycin versus placebo 12 subjects per group gave us a power of 0.95 at an alpha of 0.05 to detect a 75 ± 49 kcal/day (mean±SD) difference in stool calories.
Data exclusions	Except for impaired glucose tolerance and obesity, all participants were deemed healthy by medical history, physical exam and laboratory testing. Two individuals left the study due to personal reasons after completing the over and underfeeding portion of the study and after randomization to vancomycin or placebo. All the exclusion criteria were pre-established.
Replication	An experimental replication was not attempted for this study
Randomization	The randomization and release of the medication was managed by the pharmacy at the Phoenix Indian Medical Center using a permuted block randomization by sex with a block sizes ranging from 1 to 5
Blinding	This study was randomized double-blind placebo-controlled trial

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Human research participants

Policy information about studies involving human research participants

Population characteristics	51 individuals aged 18-45 years old (10 females) with BMI (Kg/m^2) range 32.3 ± 8.0
Recruitment	Subjects were recruited from the greater Phoenix area. The volunteers were reimbursed for the inpatient stay. No evidence of self-selection bias.
Ethics oversight	The experimental protocol was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	ClinicalTrials.gov identifier: NCT02037295
Study protocol	Available as Supplement Material
Data collection	Subjects were recruited from the greater Phoenix area from March 2014 to May 2017
Outcomes	We designed a clinical study to test whether interventions designed to alter the gut microbiota cause significant differences in stool calorie loss, which serve as an experimentally quantifiable proxy for nutrient absorption. In this two-phase study, we directly measured stool and ingested calories along with plasma biomarkers of host and microbial metabolism during over- and under-feeding (Phase I) and then in a randomized double-blind placebo-controlled trial with oral vancomycin versus placebo (Phase II).