nature portfolio

Peer Review File

Identification of gut microbiome features associated with host metabolic health in a large population-based cohort

Corresponding Author: Professor Eran Segal

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors examined the gut microbiome of over 9,000 individuals, focusing on the functional aspects of bacterial gene families and pathways, and their correlation with measures of metabolic health. They uncovered intriguing associations between certain bacterial gene families and metabolic features. However, these findings are preliminary due to significant limitations in the replication cohort, where only 49.2% of bacterial gene families showed significant replication. To validate these connections, further investigation into the causal relationship between bacteria and metabolic health measures using bioinformatic approaches or wet lab experiments is strongly recommended. Additionally, the discussion on the unexpected correlations should be more in-depth and thorough.

Reviewer #2

(Remarks to the Author)
This is an interesting study that examines microbial functional features in relation to multiple measures of metabolic health in a large population-based study. The analysis approach is straightforward, and the study only focuses on community-level functions. A few functional features were identified, and most of the functions are relatively “housekeeping” without presenting a clear link to the underlying pathogenesis of metabolic diseases. My specific comments are:
1. First of all, the introduction section implies that microbial functional analysis is not common for studying metabolic diseases and traits. However, functional analysis based on biochemical pathways, enzymes, and gene families is not rare but rather commonly seen in studies of microbiome and cardiometabolic traits, e.g., Z Mei, Nature Medicine. 2024; K Wang, Microbiome. 2023; J Li. Gut. 2022; D Wang. Nature Medicine. 2021.
2. This study focuses entirely on functional features. However, it is essential that taxonomic and functional features are analyzed together. This is because the following two scenarios are biologically interesting and can lead to different translations: 1) a function is associated with a phenotype because it is encoded by a microbe is associated with the phenotype vs. 2) community-level functional shifts, where multiple microbes encoding the function are consistently enriched/depleted in your phenotype. The authors stated they have taxonomical profiles but did not include them in the main findings. It would be useful to present the microbes that contribute to each of the functions and better distinguish the two scenarios as described.
3. Typos and grammatical errors can be found here and there. For example, “perspective” should be “prospective” in the Introduction section. Proofreading is necessary.
4. I am not sure about the meaning of the authors’ rationale statement for this study: “…their focus on individuals with metabolic disorders or the depth of the microbiome analysis, restricting the ability to generalize their findings to a broader understanding of the microbiome’s role in metabolic health…” Does this mean this current study excluded individuals with metabolic disorders? What does “the depth of the microbiome analysis” mean? Why these limitations will be critical issues for generalizability? Did this current study specifically address the generalizability?
5. It is unclear to me how the Bonferroni correction was done. Was it done across the board, i.e., taking into account all the features and all the traits? Or was it done within each trait?
6. It would be useful to specify the detailed HUMAnN3 version, as some of the HUMAnN3 versions may not be compatible with MetaPhlAn 4.0.
7. Instead of gene clumping, I suggest the authors conduct an orthogonal filtering upfront to address the high corrections between the functional features.
8. It is unclear to me how the dietary analysis was conducted. Please provide more details on that analysis incorporated

dietary data. In addition, the processing and modeling of dietary variables are suboptimal. Only using % calories from carbohydrates, proteins and lipids is not sufficient to capture dietary patterns. I would suggest the authors use factor analysis to capture multiple dietary patterns based on individual food intakes or use a comprehensive empirical dietary score such as the hPDI (health plant-based dietary index).
9. I imagine many individuals are taking medications for therapeutic and preventive purposes, dietary supplements, and pre/probiotics. How were these variables taken into account, given that medication uses, e.g., metformin, have been proven confounders in the microbiome-metabolic trait associations?
10. Fig4: It will be useful to include correlation coefficients in the figures.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have no more questions.

Reviewer #2

(Remarks to the Author)
Thanks for the authors' efforts in addressing my comments. However, I have some follow-up questions regarding the authors' responses.
Response to comment #1
The authors stated, “Yet, many of the studies to date faced limitations due to the scale of their cohort or measured phenotypes, their focus on individuals with metabolic disorders or the depth of the microbiome analysis, restricting the ability to generalize their findings to a broader understanding of the microbiome’s role in metabolic health.” Contrary to the authors' claims, the cohorts they referenced are more comprehensively phenotyped, with a focus on generally healthy participants. These cohorts employed deep-sequencing technologies and conducted thorough investigations into both the composition and functional aspects of the gut microbiome. Please update the claims.
Response to comment #2
It is puzzling that the authors only presented genus-level taxonomy and only presented the results for a few pathways. I suggest the authors present species-level results for all the pathways.
Response to comment #3
Again, those studies referenced included thorough investigations into both the composition and functional aspects of the gut microbiome and generally healthy people with more comprehensive phenotyping. Many of the studies are based on long-running epidemiological cohorts in the US with decades of experience in studying all kinds of phenotypes, biomarkers, and health conditions. Please state what is true here.
Response to comment #7
“Orthogonal filtering before testing could lead to the exclusion of features that may be significantly associated with our metabolic health measures.” This does not sound like a convincing justification for not using orthogonal filtering. Let’s say you have two functional features that are both significantly associated with a metabolic trait and highly correlated. To me, this will be very likely to reflect the fact you capture redundant biological mechanisms towards the same pathogenic pathway of a metabolic disease/condition.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Thanks for the authors' effort in addressing my comments. I have no more comments.

Reviewers' comments

Reviewer 1

In this manuscript, the authors examined the gut microbiome of over 9,000 individuals, focusing on the functional aspects of bacterial gene families and pathways, and their correlation with measures of metabolic health. They uncovered intriguing associations between certain bacterial gene families and metabolic features. However, these findings are preliminary due to significant limitations in the replication cohort, where only 49.2% of bacterial gene families showed significant replication. To validate these connections, further investigation into the causal relationship between bacteria and metabolic health measures using bioinformatic approaches or wet lab experiments is strongly recommended. Additionally, the discussion on the unexpected correlations should be more in-depth and thorough.

We appreciate the reviewer's insightful comments and agree that our results, while intriguing, require further validation and exploration in additional cohorts. As noted in the discussion, we recognize the need for using more methods to investigate their causal nature.

Following the reviewer's suggestion, we have expanded the discussion, elaborating on the predominantly negative associations observed between bacterial gene families and pathways and metabolic health measures. Additionally, we provided further detail on the "key" bacterial pathways and taxonomic contribution analysis included in this revision. We also revised the limitations section to acknowledge the need for future studies to explore the causal nature of these associations.

Reviewer 2

This is an interesting study that examines microbial functional features in relation to multiple measures of metabolic health in a large population-based study. The analysis approach is straightforward, and the study only focuses on community-level functions. A few functional features were identified, and most of the functions are relatively "housekeeping" without presenting a clear link to the underlying pathogenesis of metabolic diseases. My specific comments are:

1. First of all, the introduction section implies that microbial functional analysis is not common for studying metabolic diseases and traits. However, functional analysis based on biochemical pathways, enzymes, and gene families is not rare but rather commonly seen in studies of microbiome and cardiometabolic traits, e.g., Z Mei, Nature Medicine. 2024; K Wang, Microbiome. 2023; J Li. Gut. 2022; D Wang. Nature Medicine. 2021.

We thank the reviewer for their insightful comment and for directing us to additional relevant sources. In response, we have revised the third paragraph of the introduction to explicitly include recent studies, which are underlined in the revised section below.

“Despite the significant strides made in understanding the gut microbiome's influence on metabolic health, our knowledge remains fragmented. While studies have highlighted associations between the microbiome and metabolic factors such as glucose homeostasis and obesity [1], a comprehensive understanding of the functional mechanisms by which the microbiome influences these processes is still evolving. Recent research has begun to shed light on the metabolic pathways influenced by the gut microbiome, revealing potential links to energy metabolism, inflammation, and insulin sensitivity. Several studies have established the relation between gut microbiome composition and diversity to Short-Chain Fatty Acids (SCFAs) production, which in turn regulates several metabolic pathways, is involved in obesity, insulin resistance and T2D [2]. Gut microbiome dysbiosis has also been linked to alterations in bile acid (BA) metabolism, which is crucial for liver health [3]. Additionally, research has identified gut bacteria associated with insulin resistance and insulin sensitivity, and demonstrated that these bacteria improve host insulin resistance in mouse models [4]. Recent research has increasingly focused on the functional aspects of the gut microbiome. A study demonstrated that adherence to a Mediterranean-style diet is linked with specific functional components of the gut microbiome, potentially mediating its protective effect against cardiometabolic diseases [5]. A more recent study found an elevated abundance of functions related to bacterial cellular metabolism, particularly favoring glycolysis, in patients with type 2 diabetes [6]. Yet, many of the studies to date faced limitations due to the scale of their cohort or measured phenotypes, their focus on individuals with metabolic disorders or the depth of the microbiome analysis, restricting the ability to generalize their findings to a broader understanding of the microbiome’s role in metabolic health. ”

2. This study focuses entirely on functional features. However, it is essential that taxonomic and functional features are analyzed together. This is because the following two scenarios are biologically interesting and can lead to different translations: 1) a function is associated with a phenotype because it is encoded by a microbe is associated with the phenotype vs. 2) community-level functional shifts, where multiple microbes encoding the function are consistently enriched/depleted in your phenotype. The authors stated they have taxonomic profiles but did not include them in the main findings. It would be useful to present the microbes that contribute to each of the functions and better distinguish the two scenarios as described.

We thank the reviewer for highlighting the potential importance of considering taxonomic profiles in addition to functional features. Taxonomy information of metagenomic samples is highly dependent on the reference genomes, and the algorithms used to align the data to them - incomplete or biased reference databases could potentially influence the identification and resolution of microbial taxa [7]. Thus, we chose in this work to focus on the functional features of the gut microbiome. Nevertheless, following the questions raised by the reviewer, we have incorporated an additional analysis to the study, examining the taxonomic contributions to the “key” pathways highlighted by our analysis.

Utilizing the HUMAnN output, which provides information on the contributions of bacterial genera (when possible) - we analyzed for each “key” pathway the genera that contributed to its overall presence in the gut microbiome community. For each “key” pathway we first identified

the contributing genera, determined as genera contributing to the abundance of the pathway in any amount (RPKs > 0) in at least 500 samples. Following, we examined for each “key” bacterial pathway, the distribution of genera contributions (in RPKs). Most “key” bacterial pathways were found to be contributed by several genera, ranging from 10 up to 33. 3 “key” bacterial pathways had all their reads assigned as ‘*unclassified*’ by the HUMAnN output, suggesting we lack taxonomic resolution to assign them to a known genera, further stressing the importance of analyzing microbiome data on a functional level. We believe the results (further detailed below) strengthen our findings, as they potentially imply that the majority of the “key” bacterial pathways identified present community-level functionalities that associate with the metabolic health measures of the host.

The figures below present the distributions of contributing genera for the 11 “key” bacterial pathways, divided to three clusters based on their correlations to the metabolic health outcomes (as shown in Figure 3A). Analyzing these figures we added the following paragraph to our study results:

Community-Level Contributions to “Key” Bacterial Pathways

The "key" bacterial pathways identified in the previous analysis represent specific functionalities associated with metabolic health measures. These functionalities could result from two different biological scenarios: (1) a community-level functionality where multiple microbial species or genera contribute to an overall functional capacity, or (2) a scenario where a single microbial species or genus predominantly encodes the functionality. To distinguish between these possibilities, we utilized the HUMAnN generated abundance contributions from specific organisms to the "key" bacterial pathways. For each "key" bacterial and pathway, we identified the contributing genera (see Metagenomic Reads Mapping and Functional Profiling in Methods) and examined the distribution of their contributions in terms of RPKs.

The pathways in Cluster I exhibited a diverse contribution from multiple genera, with 10-24 genera contributing to each bacterial pathway's abundance (Figure S2). This suggests a community-level functionality where several genera are collectively responsible for the metabolic functions associated with these pathways. Similarly, the pathways in Cluster III also showed contributions from a wide range of genera, with 16-33 genera contributing to each pathway's abundance (Figure S4). This pattern further supports the concept of community-level functionality for these “key” bacterial pathways. In contrast, the bacterial pathways in Cluster II were predominantly associated with unclassified bacteria (Figure S3), meaning we currently lack sufficient taxonomic resolution to attribute these functions to specific known genera. As a result, it remains unclear whether these pathways are driven by a single microbial taxon or reflect a broader community-level functionality. Only one pathway in this cluster, PWY0-1296, had contributions from 19 recognized genera (Figure S3).

The diversity of genera contributing to these key pathways suggests that they likely represent community-level functionalities associated with metabolic health measures. Additionally, the presence of unclassified contributions for some pathways highlights the importance of analyzing gut microbiome data at the functional level, as there are still under-characterized taxonomic groups that could lead to a loss of valuable information.

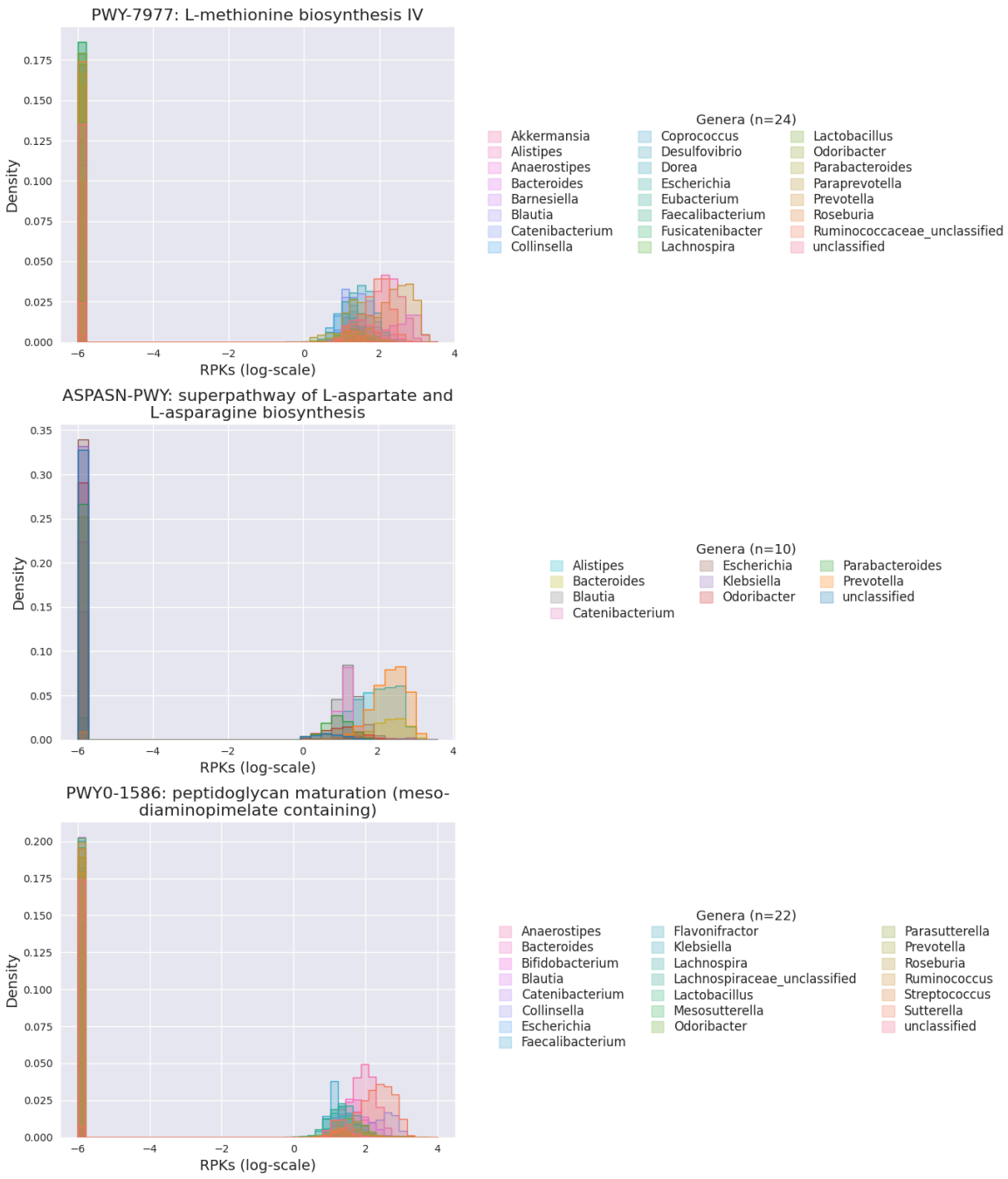

Figure S2: Genera contributing to the abundance of "Key" Pathways from Cluster I.
Histogram of RPKs (log-scale) contributed by each genera to the 3 "Key" pathways from cluster I (top to bottom): **PWY-7977, ASPASN-PWY, PWY0-1586 .** Legend beside each figure shows the number of genera that contributed to the pathway, along with its corresponding color.

Figure S3: Genera contributing to the abundance of "Key" Pathways from Cluster II.
Histogram of RPKs (log-scale) contributed by each genera to the 3 "Key" pathways from cluster I (top to bottom): **PWY0-1296, PWY-7761, PWY-7383, PWY66-399 .** Legend beside each figure shows the number of genera that contributed to the pathway, along with its corresponding color.

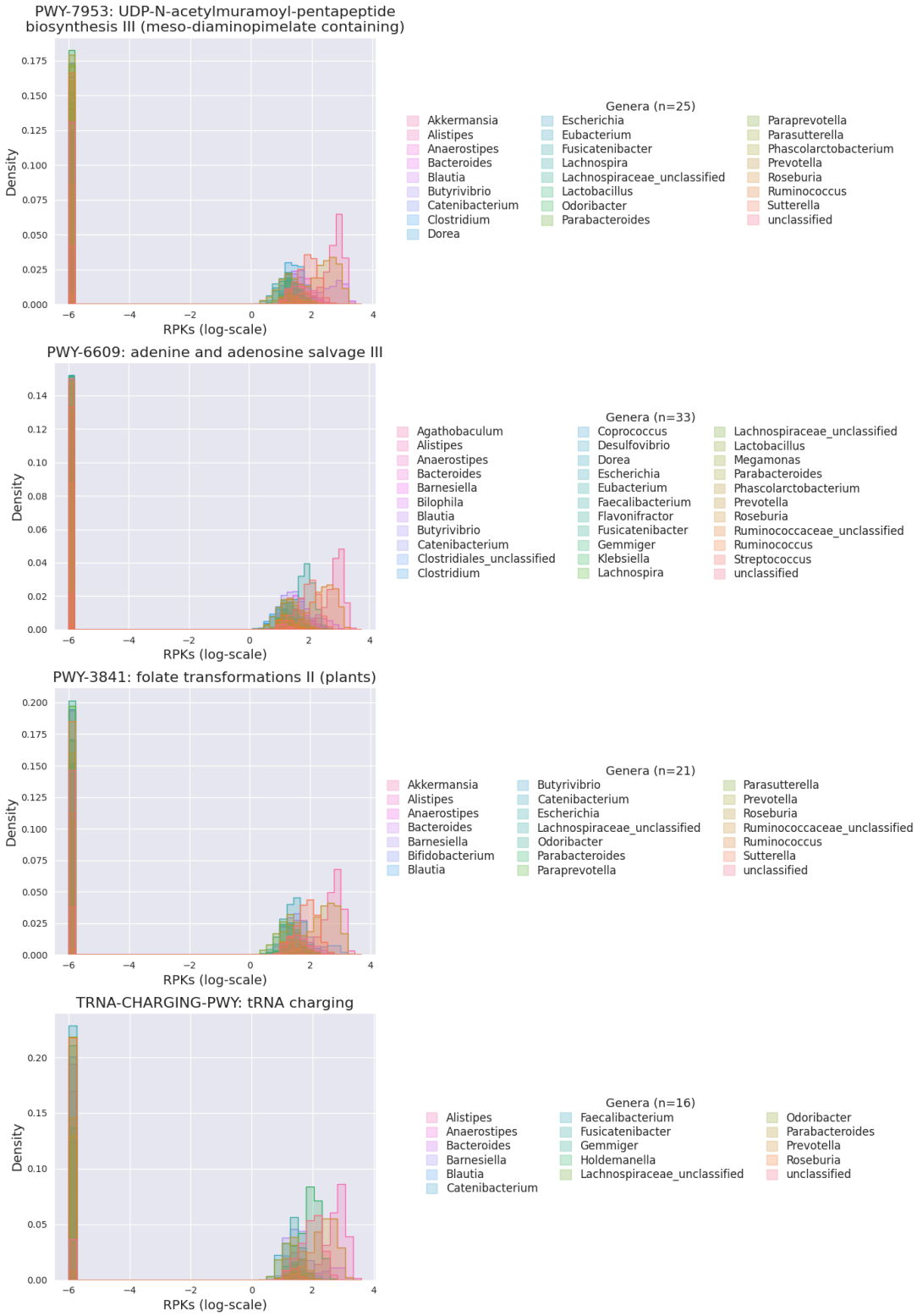

Figure S4: Genera contributing to the abundance of "Key" Pathways from Cluster III.
Histogram of RPKs (log-scale) contributed by each genera to the 3 "Key" pathways from cluster I (top to bottom): **PWY-7953, PWY-6609, PWY-3841, TRNA-CHARGING-PWY.** Legend beside each figure shows the number of genera that contributed to the pathway, along with its corresponding color.

3. Typos and grammatical errors can be found here and there. For example, "perspective" should be "prospective" in the Introduction section. Proofreading is necessary.

We thank the reviewer for bringing attention to the typos and grammatical errors present in the manuscript, including the specific example where "perspective" should have been "prospective." We have thoroughly reviewed the manuscript and corrected all identified errors to improve the clarity and accuracy of the text.

4. I am not sure about the meaning of the authors' rationale statement for this study: "…their focus on individuals with metabolic disorders or the depth of the microbiome analysis, restricting the ability to generalize their findings to a broader understanding of the microbiome's role in metabolic health…" Does this mean this current study excluded individuals with metabolic disorders? What does "the depth of the microbiome analysis" mean? Why will these limitations be critical issues for generalizability? Did this current study specifically address the generalizability?

Thank you for your comment regarding our rationale. The sentence in question was intended to emphasize that many previous studies have focused on small, often metabolically unhealthy cohorts and primarily on microbiome composition rather than functional aspects. These factors are crucial for generalizability of the results since they can bias findings towards specific populations or narrower aspects of microbiome function.

Our study aims to address these gaps by utilizing a large, predominantly healthy cohort and focusing on functional analysis, as well as incorporate sensitivity analysis and replication of results where possible. To clarify our intent, we have revised the closing sentence of the third paragraph of the introduction, which is underlined below.

Despite the significant strides made in understanding the gut microbiome's influence on metabolic health, our knowledge remains fragmented. While studies have highlighted associations between the microbiome and metabolic factors such as glucose homeostasis and obesity [1], a comprehensive understanding of the functional mechanisms by which the microbiome influences these processes is still evolving. Recent research has begun to shed light on the metabolic pathways influenced by the gut microbiome, revealing potential links to energy metabolism, inflammation, and insulin sensitivity. Several studies have established the relation between gut microbiome composition and diversity to Short-Chain Fatty Acids (SCFAs) production, which in turn regulates several metabolic pathways, is involved in obesity, insulin resistance and T2D [2]. Gut microbiome dysbiosis has also been linked to alterations in bile acid (BA) metabolism, which is crucial for liver health [3]. Additionally, research has identified gut bacteria associated with insulin resistance and insulin sensitivity, and demonstrated that these bacteria improve host insulin resistance in mouse models [4]. Recent research has increasingly focused on the functional aspects of the gut microbiome. A study demonstrated that adherence to a Mediterranean-style diet is linked with specific functional components of the gut microbiome, potentially mediating its protective effect against cardiometabolic diseases [5]. A more recent study found an elevated abundance of functions related to bacterial cellular metabolism, particularly favoring glycolysis, in patients with type 2 diabetes [6]. Yet, many of the studies to date faced

limitations due to small cohort sizes or measured phenotypes, a focus on individuals with metabolic disorders or an emphasis on microbiome composition rather than functional analysis. These limitations possibly restrict the ability to generalize their findings to a broader and healthier population, thereby hindering a comprehensive understanding of the microbiome's role in metabolic health.

5. It is unclear to me how the Bonferroni correction was done. Was it done across the board, i.e., taking into account all the features and all the traits? Or was it done within each trait?

We thank the reviewer for this question, and see how the current phrasing might be unclear. To clarify, the Bonferroni correction was applied separately for bacterial pathways and gene families. For each category, we considered all p-values across all metabolic health outcomes collectively. We have revised the *Bacterial Gene Families\Pathways - Metabolic measures associations* under the *Methods* section to make this distinction clearer:

"Bonferroni correction was applied separately for bacterial gene families and pathways, considering all metabolic health measures collectively within each category. Specifically, the correction was performed on the entire set of p-values from the analysis of pathways across all outcomes, and this process was repeated independently for gene families. Correlations with Bonferroni-corrected p-values < 0.05 were considered statistically significant."

6. It would be useful to specify the detailed HUMAnN3 version, as some of the HUMAnN3 versions may not be compatible with MetaPhlAn 4.0.

We thank the reviewer for noting this, and we have updated the paragraph describing the process of obtaining metagenomic gene and pathway abundance data, under the section *Metagenomic reads mapping and functional profiling* (changes underlined)*;*

"Metagenomic gene and pathway abundance data were obtained using HUMAnN v3.6.1 [8] and MetaPhlAn v4.0.6 [9] utilizing the Jan21 MetaPhlAn database, and the UniRef database [10]. Genes and pathways detection thresholds were determined based on the first percentile of all positive, non-zero abundance values across the dataset, derived from HUMAnN3 outputs. *UNMAPPED* reads and *UNINTEGRATED* reads were excluded from threshold determinations for genes and pathways, accordingly. Zero abundance values were adjusted to half the detection threshold value. Abundances were sum-normalized, followed by scaling by a factor of 1,000,000 to achieve parts per million and a log10 transformation."

7. Instead of gene clumping, I suggest the authors conduct an orthogonal filtering upfront to address the high corrections between the functional features.

Thank you for your thoughtful suggestion. We understand the importance of addressing high correlations between functional features. However, in the context of our study, we perform individual OLS models for each bacterial gene family, adjusted for age and sex, to identify significant associations.

Orthogonal filtering before testing could lead to the exclusion of features that may be significantly associated with our metabolic health measures. Instead, we have employed gene clumping, a method commonly used in both GWAS (Genome-Wide Association Studies) and MWAS (Metagenomic-Wide Association Studies) [11 12 13] . Gene clumping allows us to retain correlated features while addressing redundancy post hoc, ensuring that potentially significant associations are not discarded prematurely. This approach has been widely accepted in the literature for its ability to manage the balance between feature redundancy and maintaining significant associations.

We hope this clarifies our rationale and methodology, and we appreciate your understanding.

8. It is unclear to me how the dietary analysis was conducted. Please provide more details on that analysis incorporating dietary data. In addition, the processing and modeling of dietary variables are suboptimal. Only using % calories from carbohydrates, proteins and lipids is not sufficient to capture dietary patterns. I would suggest the authors use factor analysis to capture multiple dietary patterns based on individual food intakes or use a comprehensive empirical dietary score such as the hPDI (health plant-based dietary index).

We thank the reviewer for this comment.
The dietary analysis was conducted as follows; we first assured the quality of the dietary data by removing single reported items with more than 5,000 calories, as well as days in which less than 500 calories were reported. Following, logging of each day was aggregated to obtain a daily summary of the amount of calories consumed from carbohydrates, proteins and lipids, and the total calories (kcal) consumed. Then, for each day we calculated the percent of caloric intake which originated from carbohydrates, proteins and lipids, and summarized the median of these values over all days, along with the median daily total caloric intake. These summaries were then added to our OLS models for each bacterial gene family and bacterial pathway as additional covariates. Following the reviewer's comment, we clarified these processes in the section *Nutritional summaries as sensitivity analysis* (see underlined below).

Additionally, following the reviewers suggestions to use comprehensive empirical dietary scores we added an additional covariate - the Healthy Food Diversity (HFD) Index, which measures dietary variety and quality [14]. This was added along with the original nutritional summaries as a covariate to the OLS model in our sensitivity analysis. Sensitivity analysis results remained mostly unchanged after the addition of the HFD Index as an additional covariate - as in the original analysis, we saw a large portion of bacterial gene associations (68.1%) and bacterial pathway associations (62.53%) replicated in the sensitivity analysis adjusting for nutritional summaries. As before, the main changes observed were a decrease in the number of significant associations for variability measures obtained from CGM, which aligns with prior findings that highlighted the correlations between nutritional summaries and CGM variability measures [15]. Measures of body composition retained almost all their significant associations, with Android tissue fat (%) retaining the highest number of associations with bacterial gene families - 53,186 significant associations, replicating 80.2% of the significant associations found in the main analysis, and BMI retaining the highest number of associations with bacterial pathways - 99

significant associations, replicating 98.1% of the significant associations found in the main analysis

Below see underlined the changes to the Methods section *Nutritional summaries as sensitivity analysis* which reflect the changes described above;

To analyze the potential confounding of nutritional habits on the associations between bacterial gene families\pathways and metabolic health measures, we repeated the association analysis, adjusting for summaries of nutritional habits. Using data from continuous real-time food intake logging that was reported by participants while wearing the CGM, we created summaries of nutritional habits. To assure quality control of the data we first removed individual loggings of food items containing more than 5,000 calories, and days in which less than 500 calories were reported. Following, we aggregated loggings from the same date to create a daily summary of the amount of calories consumed from carbohydrates, proteins and lipids, and the total calories (kcal) consumed. For each day we calculated the percent of calories consumed from carbohydrates, proteins and lipids, and summarized the median of these over all days, along with the median total caloric intake over all days. Additionally, the Healthy Food Diversity (HFD) Index, which measures dietary variety and quality [14] was calculated by determining the proportion of each food in the diet, multiplying by health factors, and computing the Berry Index. The HFD Index was normalized by the maximum index value in the study population. With the nutritional summaries calculated for each participant, we repeated the association process described in the *Bacterial Gene Families\Pathways - Metabolic measures associations* section, adjusting each OLS model for age and sex as well as all nutritional summaries; HFD Index, median daily caloric intake, and median daily percent of caloric intake from carbohydrates proteins and lipids.

9. I imagine many individuals are taking medications for therapeutic and preventive purposes, dietary supplements, and pre/probiotics. How were these variables taken into account, given that medication uses, e.g., metformin, have been proven confounders in the microbiome-metabolic trait associations?

Medication intake in the Human Phenotype Project (HPP) is self-reported by the participants. Following this important remark from the reviewer, we analyzed medication reports of the study population and found that a small number of participants (< 100) reported taking diabetes related medications (medications starting with 'A10' in ATC code). We removed these participants from the study population, adding this exclusion to the *Study Population* section in *Methods* (see underlined below), and re-analyzed the resulting population of 8,859 individuals. Population characteristics mainly remained unchanged (which can be seen by the updated Table 1 and Table S1 in the updated manuscript and supplementary appendix). Additionally, the main results also remained mostly unchanged as can be seen by the correlation patterns, and the "key" bacterial pathways uncovered which are identical to those in the original analysis, before the removal of this small number of participants.

Study population:

The cohort analyzed in this study was collected as part of the Human Phenotype Project (HPP). A complete description of the inclusion and exclusion criteria to the study, the measures obtained and the measurement techniques can be found in the original paper describing the HPP [16]. The study includes individuals between the age of 40-70 years old. Data collected at baseline includes self-reported sex, medical history, lifestyle and nutritional habits, vital signs, anthropometrics, metagenomic sequencing of the gut microbiome, blood tests results, electrocardiography, carotid ultrasound, liver US, Dual-energy X-ray absorptiometry (DXA) scan and retinal imaging. Continuous measurements include glucose levels using a CGM device for 2 weeks, along with real-time food intake loggins and sleep monitoring by a home sleep apnea test device for 3 nights. In this study we included participants with both CGM and gut-microbiome data, excluding participants who reported taking diabetes related medications (medications starting with A10 in ATC code), which resulted in 8,859 participants, of them 4,778 (53.9%) women and 4,081 (46.1%) men, with an average age of 51.8 (7.8) years and average BMI of 26.01(4.1).

10. Fig4: It will be useful to include correlation coefficients in the figures.

We thank the reviewer for this comment, and following it we added the Pearson correlation for both the coefficients and the p-values shown in Figure 4, as well as Figure S4. The updated Figure 4 can be seen below.

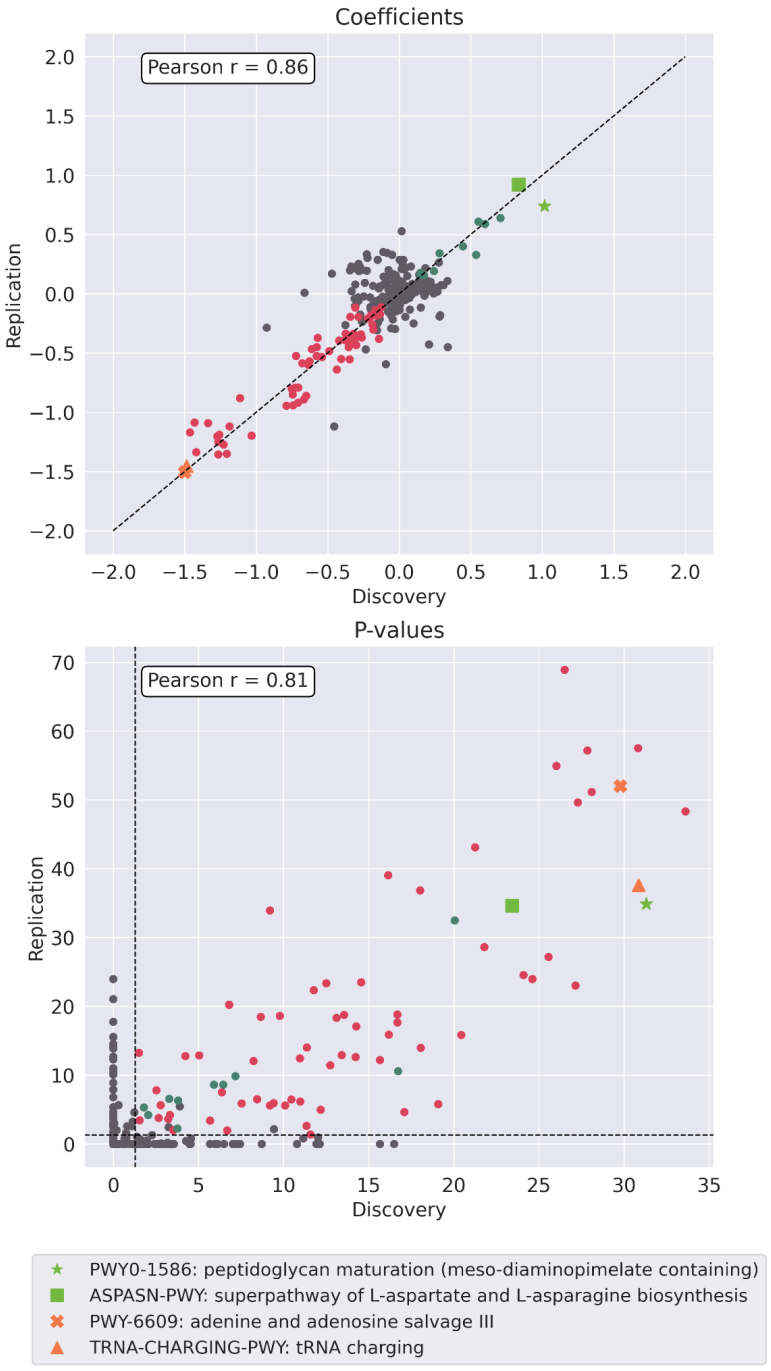

Figure 4: Bacterial Pathways correlate with BMI replicate in an independent cohort.
Coefficients (top) and **P-values (-log scale) (bottom)** of the associations between bacterial pathways and BMI, in the discovery vs. the replication cohort. Colored dots present bacterial pathways with significant Bonferroni-corrected p-values (<0.05) in both cohorts, and with either a negative (red) or positive (green) correlation coefficient in both cohorts. Light green (Square and Asterix) mark the top two bacterial pathways with positive coefficients in both cohorts, Orange (Triangle and X) mark the top two bacterial pathways with negative coefficients in both cohorts.

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Reviewers' comments

Reviewer 2

Response to comment #1
The authors stated, "Yet, many of the studies to date faced limitations due to the scale of their cohort or measured phenotypes, their focus on individuals with metabolic disorders or the depth of the microbiome analysis, restricting the ability to generalize their findings to a broader understanding of the microbiome's role in metabolic health." Contrary to the authors' claims, the cohorts they referenced are more comprehensively phenotyped, with a focus on generally healthy participants. These cohorts employed deep-sequencing technologies and conducted thorough investigations into both the composition and functional aspects of the gut microbiome. Please update the claims.

We appreciate the reviewer's thoughtful feedback. In response, we have revised the introduction to acknowledge that several of the referenced studies were indeed conducted in well-phenotyped cohorts with detailed investigations into both the composition and functional aspects of the gut microbiome. We agree that these studies have provided significant insights into the relationship between the microbiome and metabolic health. We intended to highlight that, in some cases, smaller cohort sizes or a focus on specific populations (e.g., individuals with metabolic disorders) may limit the generalizability of these findings to healthier populations. We have updated the wording to reflect this, and now also reference research by Rothschild et al. (2022), which demonstrates the importance of large cohort sizes for robust microbiome associations with host phenotypes. Below is the revised section of the introduction (underlined):

"A study demonstrated that adherence to a Mediterranean-style diet is linked with specific functional components of the gut microbiome, potentially mediating its protective effect against cardiometabolic diseases 21. A more recent study found an elevated abundance of functions related to bacterial cellular metabolism, particularly favoring glycolysis, in patients with type 2 diabetes 22. Yet, many of the studies to date faced limitations due to small cohort sizes or measured phenotypes, a focus on individuals with metabolic disorders or an emphasis on microbiome composition rather than functional analysis. These limitations possibly restrict the ability to generalize their findings to a broader and healthier population, thereby hindering a comprehensive understanding of the microbiome's role in metabolic health.

While previous studies, some conducted in well-phenotyped cohorts, and investigated both the composition and functional aspects of the gut microbiome [1] [2], provided valuable insights, we are still far from understanding the role of the gut microbiome in host metabolic health. Previous research shows that large cohort sizes are crucial for accurately capturing associations between the microbiome and host phenotypes [3]. Expanding research to large, predominantly healthy populations can enhance the generalizability of findings and contribute to a more comprehensive understanding of the microbiome's role in metabolic health."

Response to comment #2
It is puzzling that the authors only presented genus-level taxonomy and only presented the results for a few pathways. I suggest the authors present species-level results for all the pathways.

Following the reviewers suggestion, we have incorporated both genus and species level results for all bacterial pathways, and for the "key" bacterial pathways.

Overall, out of the 346 bacterial pathways that were associated with metabolic health measures, 74 (21.4%) pathways were annotated as being contributed from unclassified bacteria, while the majority of the pathways (272 (78.6%)) were annotated to be contributed from known bacterial species and genera. The majority of the pathways, 231 (66.8%), were found to have multiple species contributing to their abundances. Figure S2 (added below) shows the distributions of the number of contributing genera and species to the abundance of each of the bacterial pathways (excluding pathways that were contributed solely from unclassified bacteria).

For the 11 "key" bacterial pathways, we also added figures displaying the distributions of contributing species, divided to three clusters based on their correlations to the metabolic health outcomes, similar to the genera contributing distribution figures (added below).

We have updated the section *Community-Level Contributions to Bacterial Pathways* of the results to include these additional analyses (changes are underlined below).

The "key" bacterial pathways identified in the previous analysis represent specific functionalities associated with metabolic health measures. These functionalities could result from two different biological scenarios: (1) a community-level functionality where multiple microbial species or genera contribute to an overall functional capacity, or (2) a scenario where a single microbial species or genus predominantly encodes the functionality. To distinguish between these possibilities, we utilized the HUMAnN generated abundance contributions from specific organisms [4] to the abundance of bacterial pathways. For each bacterial pathway, we identified the contributing genera and species (see Metagenomic Reads Mapping and Functional Profiling in Methods) and examined the distribution of their contributions in terms of RPKs.

Of the 346 bacterial pathways associated with metabolic health measures, 74 (21.4%) were attributed to unclassified bacteria, while the majority, 272 (78.6%), were linked to known bacterial species and genera. The number of contributing genera and species varied across bacterial pathways (Figure S2), with an average of 13.02 species (±20.03) and 7.4 genera (±10.05) contributing to the abundance of each bacterial pathway. Notably, most pathways (231, or 66.8%) had their abundances contributed by multiple species.

The pathways in Cluster I exhibited a diverse contribution from multiple genera, with 10-24 genera, and 22-53 species contributing to each bacterial pathway's abundance (Figures S~~2~~3, S4). This suggests a community-level functionality where several genera are collectively responsible for the metabolic functions associated with these pathways. Similarly, the pathways in Cluster III also showed contributions from a wide range of genera and species, with 16-33 genera and 40-65 species contributing to each pathway's abundance (Figures S7, S8). This pattern further supports the concept of community-level functionality for these "key" bacterial pathways. In contrast, the bacterial pathways in Cluster II were predominantly associated with unclassified bacteria (Figures S~~3~~5, S6), meaning we currently lack sufficient taxonomic resolution to attribute these functions to specific known genera or

species. As a result, it remains unclear whether these pathways are driven by a single microbial taxon or reflect a broader community-level functionality. Only one pathway in this cluster, PWY0-1296, had contributions from 19 recognized genera and 23 recognized species (Figures S35, S6).

The variable distributions of the number of contributing genera and species, and the diversity of genera and species contributing to the "key" pathways suggests that these likely represent community-level functionalities associated with metabolic health measures. Additionally, the presence of unclassified contributions for some pathways highlights the importance of analyzing gut microbiome data at the functional level, as there are still under-characterized taxonomic groups that could lead to a loss of valuable information.

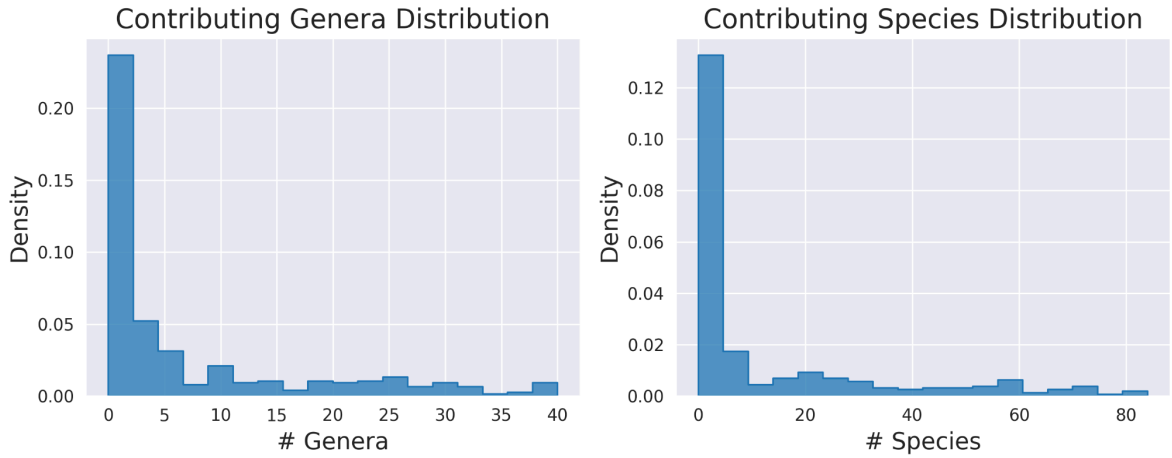

Figure S2: Number of Contributing Genera and Species to the abundance of bacterial pathways. Distribution plots show the density of the number of contributing **Genera (Left)** and **Species (Right)** to the abundances of the analyzed bacterial pathways. Pathways for which all abundances were from unclassified genera were excluded from the distribution.

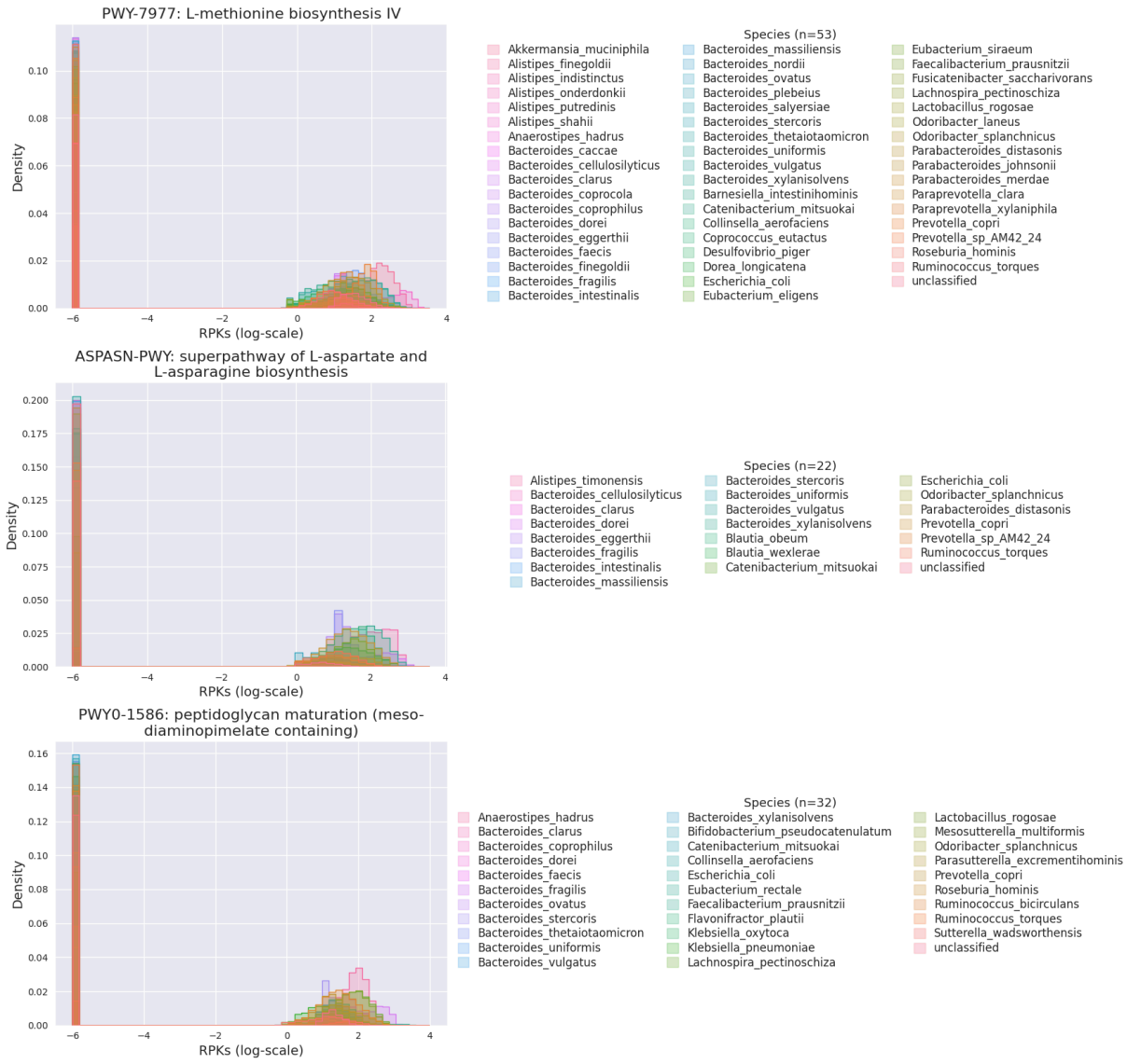

Figure S4: Species contributing to the abundance of "Key" Pathways from Cluster I.
Histogram of RPKs (log-scale) contributed by each species to the 3 "Key" pathways from cluster I (top to bottom): **PWY-7977, ASPASN-PWY, PWY0-1586 .** Legend beside each figure shows the number of species that contributed to the pathway, along with its corresponding color.

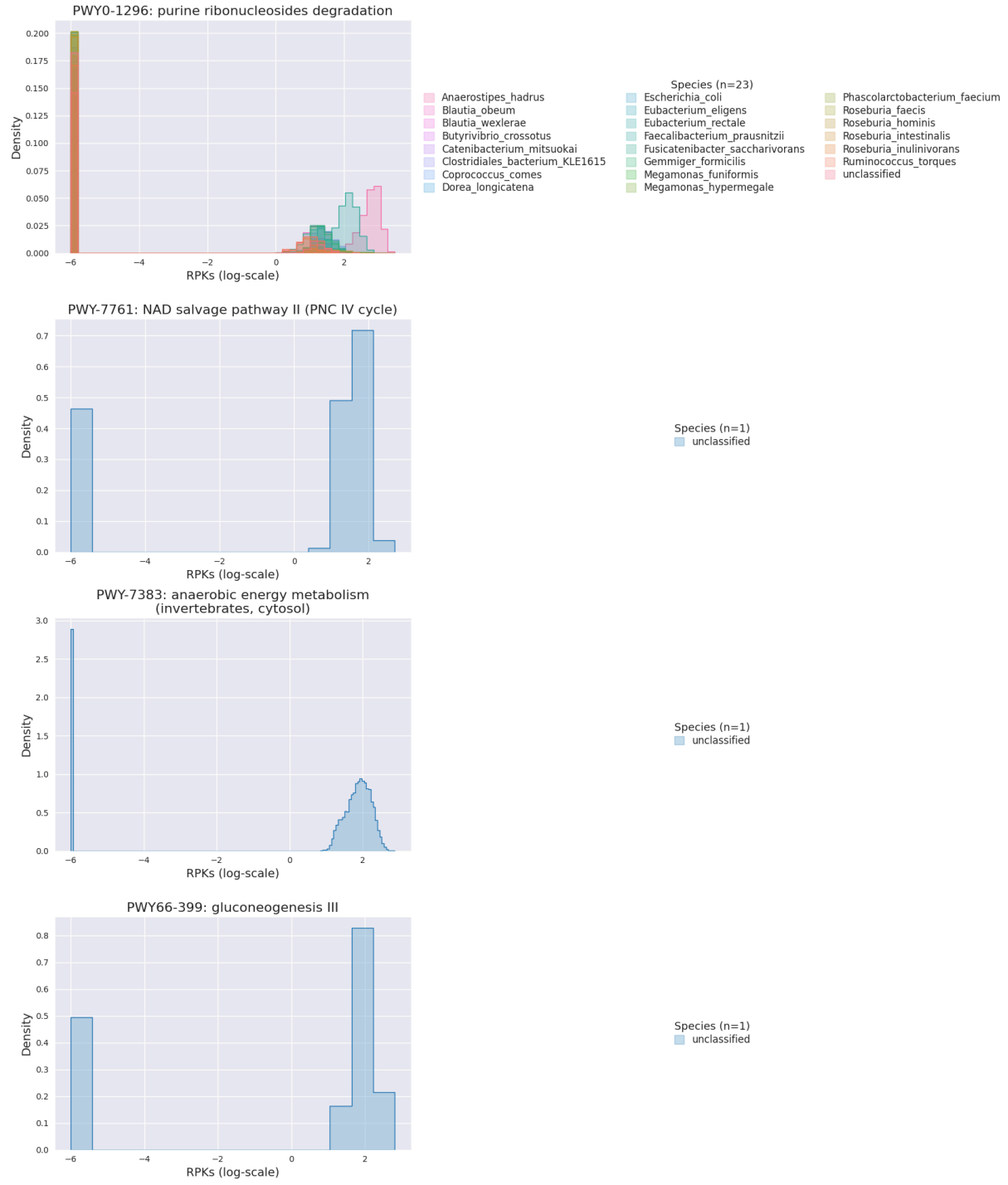

Figure S6: Species contributing to the abundance of "Key" Pathways from Cluster II.
Histogram of RPKs (log-scale) contributed by each species to the 3 "Key" pathways from cluster I (top to bottom): **PWY0-1296, PWY-7761, PWY-7383, PWY66-399 .** Legend beside each figure shows the number of species that contributed to the pathway, along with its corresponding color.

Figure S8: Species contributing to the abundance of "Key" Pathways from Cluster III.
Histogram of RPKs (log-scale) contributed by each species to the 3 "Key" pathways from cluster I (top to bottom): **PWY-7953, PWY-6609, PWY-3841, TRNA-CHARGING-PWY .** Legend beside each figure shows the number of species that contributed to the pathway, along with its corresponding color.

Response to comment #3
Again, those studies referenced included thorough investigations into both the composition and functional aspects of the gut microbiome and generally healthy people with more comprehensive phenotyping. Many of the studies are based on long-running epidemiological cohorts in the US with decades of experience in studying all kinds of phenotypes, biomarkers, and health conditions. Please state what is true here.

We acknowledge that these studies included thorough investigations into both the composition and functional aspects of the gut microbiome, and many were based on well-established epidemiological cohorts with comprehensive phenotyping. To more accurately reflect these strengths, we have revised the introduction following the feedback from this comment and *Comment #1*. We now also reference the findings of Rothschild et al. (2022) [3], which emphasize the importance of large cohort sizes for ensuring reliable and generalizable microbiome associations with host phenotypes. Below is the revised section of the introduction (underlined):

"A study demonstrated that adherence to a Mediterranean-style diet is linked with specific functional components of the gut microbiome, potentially mediating its protective effect against cardiometabolic diseases 21. A more recent study found an elevated abundance of functions related to bacterial cellular metabolism, particularly favoring glycolysis, in patients with type 2 diabetes 22. Yet, many of the studies to date faced limitations due to small cohort sizes or measured phenotypes, a focus on individuals with metabolic disorders or an emphasis on microbiome composition rather than functional analysis. These limitations possibly restrict the ability to generalize their findings to a broader and healthier population, thereby hindering a comprehensive understanding of the microbiome's role in metabolic health.

While previous studies, some conducted in well-phenotyped cohorts, and investigated both the composition and functional aspects of the gut microbiome [1 2] , provided valuable insights, we are still far from understanding the role of the gut microbiome in host metabolic health. Previous research shows that large cohort sizes are crucial for accurately capturing associations between the microbiome and host phenotypes [3]. Expanding research to large, predominantly healthy populations can enhance the generalizability of findings and contribute to a more comprehensive understanding of the microbiome's role in metabolic health."

Response to comment #7
"Orthogonal filtering before testing could lead to the exclusion of features that may be significantly associated with our metabolic health measures." This does not sound like a convincing justification for not using orthogonal filtering. Let's say you have two functional features that are both significantly associated with a metabolic trait and highly correlated. To me, this will be very likely to reflect the fact you capture redundant biological mechanisms towards the same pathogenic pathway of a metabolic disease/condition.

In response to the reviewers feedback, we have added an additional analysis to conduct upfront gene filtering. We applied pairwise Spearman correlations followed by hierarchical clustering to identify independent bacterial gene families prior to running our associations with metabolic health measures. This filtering process yielded 55,925 pre-filtered gene representatives with a

weighted mean correlation of 0.01 and a pooled standard deviation of 0.03. Full details of this methodology have been included in the newly added *"Upfront Gene Filtering"* section in the Methods, which is also provided below.

Upon comparing the results from this upfront filtering method with our original clumping approach, we found that both methods resulted in a similar number of significant associations across various metabolic health measures. This includes associations with android fat tissue and visceral adipose tissue mass, as well as glucose variability measures from continuous glucose monitoring (CGM). Additionally, similar correlation patterns were observed when defining "key" bacterial gene families with both methods.

These new results, along with the added figures to the Supplementary Appendix, have been included in the marked manuscript and the marked Supplementary Appendix, and are also provided below with changes underlined. We believe that the integration of both the clumping and upfront filtering approaches, which yielded consistent outcomes, strengthens the findings presented in our manuscript.

Upfront Gene Filtering

Upfront gene filtering was applied to identify uncorrelated bacterial gene families prior to running the associations with metabolic health measures. Bacterial gene families were randomly divided into groups of up to 5,000. Within each group, pairwise Spearman correlations were calculated, and hierarchical clustering was applied to the resulting distance matrix using average linkage clustering with a correlation threshold of 0.3. For each cluster, the gene with the highest average abundance was selected as the representative. This process was repeated three times to further refine the gene representatives and minimize feature redundancy, resulting in 12 groups of ~5,000 bacterial gene representatives in each group. To derive a final set of representatives, each pair from the 12 groups was re-processed by calculating Spearman correlations and applying hierarchical clustering with the same correlation threshold of 0.3. The final list of filtered bacterial gene families was defined as the unique set of representatives from all pairs of the 12 groups. Additionally, the weighted mean correlation and pooled standard deviation were computed based on the correlations within each group pair. The final list of bacterial genes included 55,925 bacterial genes, with a weighted mean correlation of 0.01 and a pooled standard deviation of 0.03.

Bacterial Gene Families and Pathways associate with metabolic health measures

To address high correlations between bacterial gene families, and identify independent bacterial gene families associated with the examined metabolic health measures, two approaches were implemented. Upfront gene filtering was employed prior to the association analysis to obtain uncorrelated representative gene families (see Upfront Gene Filtering in Methods), resulting in 55,925 pre-filtered bacterial gene families that were associated with the metabolic health measures. Clumping, commonly used in GWAS analyses (see Gene Clumping in Methods), was employed post association analysis, to each metabolic health measure individually, to obtain a subset of independent associations. Both approaches resulted in similar numbers of significant uncorrelated bacterial gene families associated with metabolic health measures (Figures S1,2). In both, Android fat tissue (%) was the measure with the highest number of independent bacterial gene families associations (1,342 from clumping, 2217 from

pre-filtering), followed by Scanned visceral adipose tissue (VAT) mass (1,317 from clumping, 2013 from pre-filtering) (Figures S1,2). While measures of short-term CGM variability, had hundreds or tens of independent associations with bacterial gene families, for example SD.wsh (148 from clumping, 140 from pre-filtering) and SD.Roc (87 from clumping, 78 from pre-filtering), measures of overall variability, within-day variability and between-day variability were found to have only several to tens of independent associations. The IQR and MAD were associated with 2,967 and 1,231 bacterial gene families accordingly. Clumping identified only 14 and 11 independent clumps of bacterial gene families (Figure S1), and pre-filtering identified 45 and 18 uncorrelated bacterial gene families (Figure S2).

"Key" associated Bacterial Gene Families and Pathways

"Key" pre-filtered bacterial gene families showed slightly stronger associations to metabolic health measures than those of the "key" clumped gene families (Figure 3B, S3), with a single correlation pattern of mostly negative correlations and positive correlations only to hypoglycemia measures derived from CGM (Figure S3). Cluster II encompasses a broad spectrum of gene families, including those with recognized enzymatic roles in metabolism such as Pyridoxamine 5'-phosphate oxidase family protein [5] and Aminotransferase, which previous research highlighted as potentially involved in the pathogenesis of metabolic syndrome [6]. Several of the "key" bacterial gene families in the clumping analysis and pre-filtering analysis were uncharacterized proteins sourced from the species *Faecalibacterium prausnitzii* (Figures 3B, S3); one of the most abundant bacterial species in the colon of healthy humans. Changes in *Faecalibacterium prausnitzii* abundances were related to various intestinal and metabolic diseases [7].

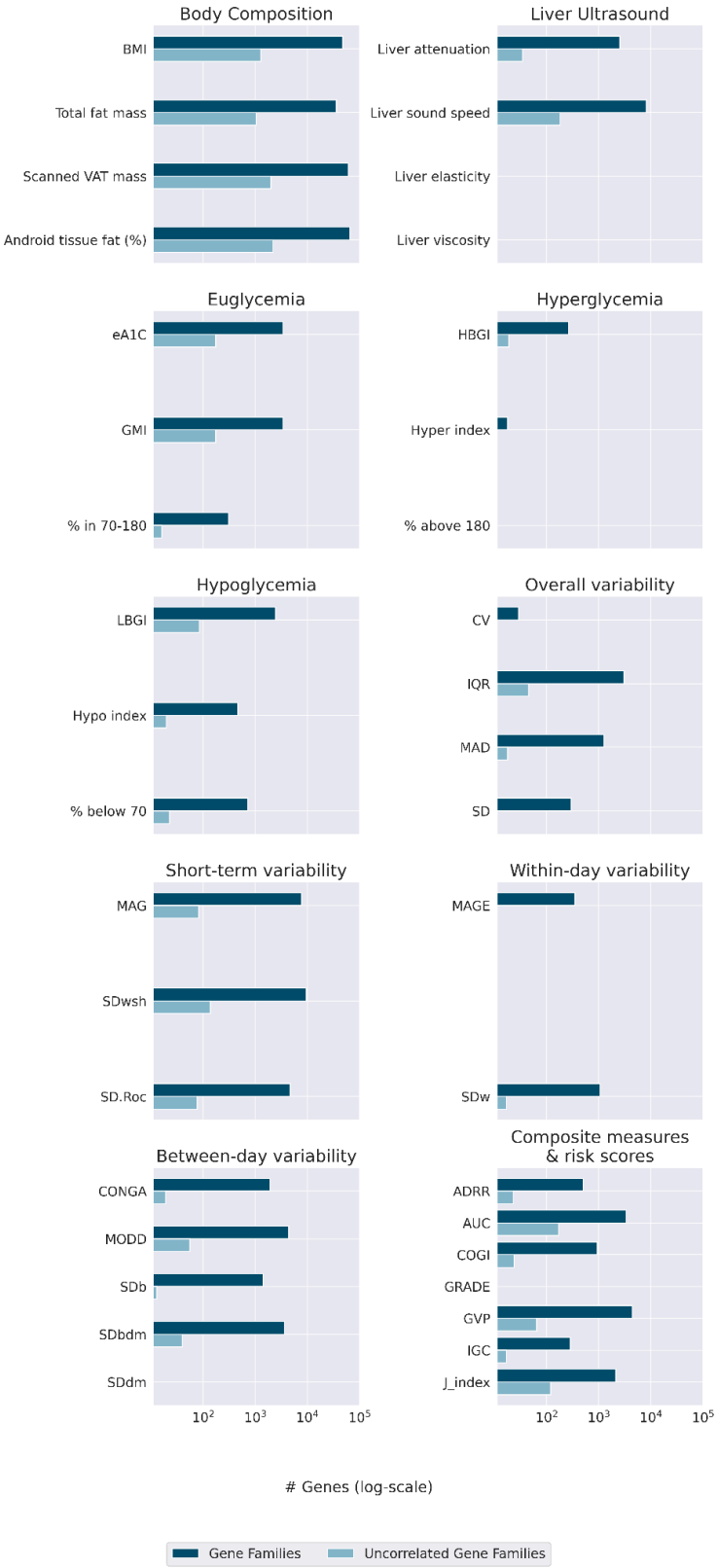

Figure S2: Number of Significantly Associated Bacterial Gene Families and Pre-Filtered Independent Bacterial Gene Families with metabolic health measures. Subplost present metabolic health measures in each category; Body composition, Liver US, Euglycemia, Hyperglycemia, Hypoglycemia, Overall variability, Short-term variability, Within-day variability, Between-day variability, Composite measures & risk scores. Bars show the number of significantly correlated bacterial gene families (dark blue) and pre-filtered independent bacteria

Table S4. "Key" Bacterial Gene Families (Pre-Filtered) - Metabolic health group selection and Hierarchical clustering

Gene Family	Body composition	Liver US	CGM-derived Measures	Cluster
Uncharacterized protein | Faecalibacterium prausnitzii			X	I
Uncharacterized protein | Faecalibacterium prausnitzii M21/2			X	
DUF3847 domain-containing protein			X	II
Stage V sporulation protein S (SpoVS)	X			
Pyridoxamine 5'-phosphate oxidase family protein	X			
Mutator family transposase	X	X		
Aminotransferase		X		
Molecular chaperone		X		
Uncharacterized protein | Faecalibacterium duncaniae		X		
CBM-cenC domain-containing protein	X	X		
Uncharacterized protein | Faecalibacterium prausnitzii M21/2			X	
Uncharacterized protein | Faecalibacterium prausnitzii			X	
Uncharacterized protein	X			III

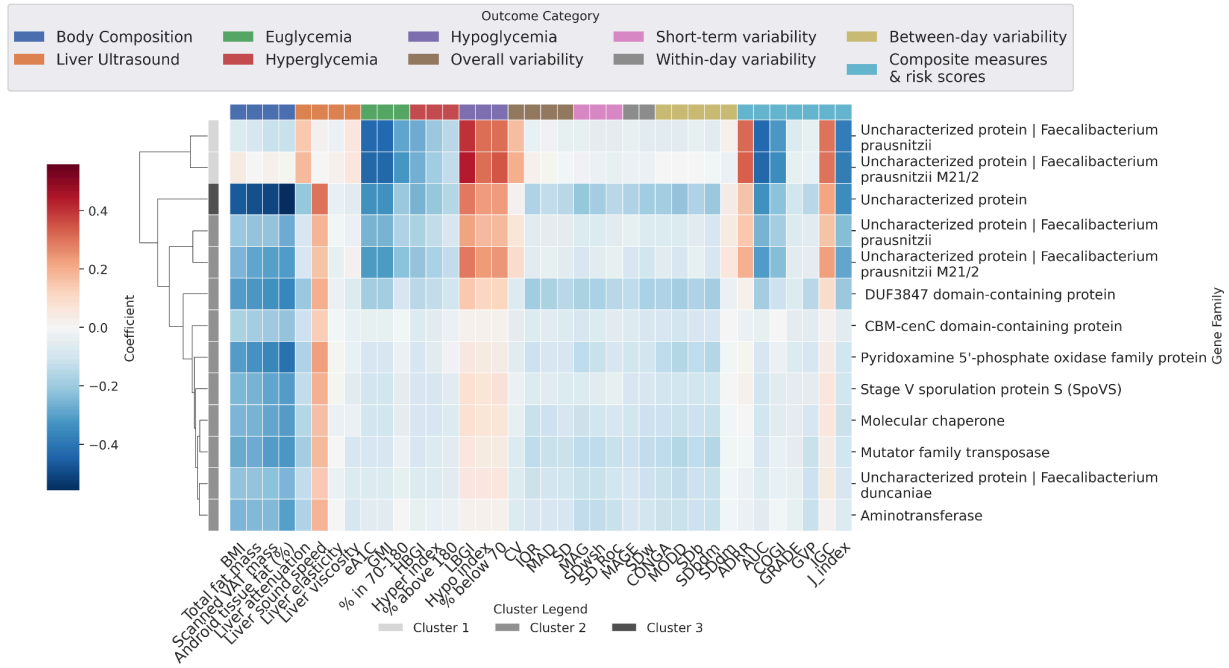

Figure S3: Heatmap of "Key" Pre-filtered Bacterial Gene Families. Heatmaps showing the correlation coefficient of the 13 "Key" Pre-filtered Bacterial Gene Families with the highest number of significantly associated metabolic health measures and lowest p-value ranking across groups (see Selecting "Key" associated Bacterial Gene Families and Pathways in Methods).

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2. Mei, Z. *et al.* Strain-specific gut microbial signatures in type 2 diabetes identified in a cross-cohort analysis of 8,117 metagenomes. *Nat. Med.* **30**, 2265–2276 (2024).
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