

The Interplay of Sleep Characteristics with Health factors and Gut Microbiome

Corresponding Author: Dr Daria Zhernakova

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)

Wu et al. addressed the relationship between self-reported sleep characteristics and gut microbiota through a large-scale microbiota association analysis, and also conducted a comprehensive mediation analysis between diet, sleep, and microbial species.

So far, the association between sleep and gut microbiome is still understudied, not to mention the potential influence of various confounding factors (e.g., mental disorders). The present study involved more than 250 phenotypes and ultimately identified that dietary factors may be closely related to both sleep and gut microbiome, thus performed further in-depth mediation analyses in a cross-sectional database. Although there is still a long way to go before interventional trials, overall, these findings may help to understand how to combine diet and the gut microbiome to improve sleep health.

The paper is in general clear and well-written. I have, however, some relevant comments and there are few questions and points to be clarified.

Major comments:

Indeed, while the authors chose dietary factors (also an intervenable factor) to understand the causal relationship between sleep and the gut microbiome, I still think it's important to address other factors that are highly correlated with sleep traits in mediation analyses, such as gastrointestinal discomfort and mental - depression. With such a large dataset of sleep-microbiome, this is a great and unique opportunity to disentangle the complex relationships among them.

Also, for the mediation analyses, the authors concentrated on 137 species, how about the overall microbial composition (beta-diversity, e.g., using principal components). And for the data presentation, since the authors have involved three potential directional relationships, it would be desirable to visualize the main findings more clearly in the figure, such as the Figure 4d, which may help the readers to quickly capture several key mediation pathways (what are exposures, mediators and outcomes).

The mediating effect, although significant, remains questionable as to its clinical implications. For example, line 295, "By aggregating the effects of UC5_1_1E11 and SGB14844, we discovered that the abundance of these species mediated 10% of the effect of coffee intake on social jet lag." Does this represent mainly a direct effect of coffee on social jet lag? How do we interpret the 10% mediation effect in this scenario?

The PSQI, as the authors mentioned, is a global sleep quality scale that includes different components, some of which actually overlap with other scales (e.g., the ESS and the PSQI-Q7). It would be interesting to look at the associations of gut microbiome and component scores, e.g., sleep duration & efficiency, which would provide a more comprehensive picture of the sleep-microbiome relationships.

In addition, are gut microbial characteristics different across subgroups? E.g., among three chronotypes, as well as those with good and poorer sleep quality

Other minor comments:

In the abstract, line 15-16, "We found that alpha diversity was inversely associated with sleep quality, chronotype, and social

jet lag...”, either rephrased as “was associated with” or specify: “was inversely associated with poorer sleep quality, earlier chronotype..”.

In methods, line 135-136, “To focus on the most relevant species, we filtered out species with a mean relative abundance present in fewer than 100 participants, leaving 813 species for further analysis.” – it is unclear, whether the authors filtered out species occurring in fewer than 100 participants (? %), or did they filter out species below a certain average relative abundance? Or both?

In discussion, line 379-380, “When considering diet as an exposure, we identified some connections that suggest a direct causal link between sleep patterns and variations in species abundance”, do the authors mean consider diet as a mediator?

Reviewer #2

(Remarks to the Author)

The manuscript presents a comprehensive analysis of the bidirectional relationship between sleep characteristics and gut microbiome composition in a large-scale Dutch cohort. The integration of various statistical methods, metagenomic sequencing that ensures detailed taxonomic profiling at the species level, improving our understanding of the complex interplay between sleep and gut microbiome composition. The identification of specific bacterial species associated with distinct sleep parameters is particularly interesting. However, some areas, such as the analysis methods and interpretation of results, require further clarification and additional analysis to strengthen the conclusions. Our specific comments are:

1. Lines 241-243: The authors report a "consistent directional trend" across species associated with positive sleep characteristics. This interpretation as a biologically meaningful finding is questionable. Instead, the trend could be due to the use of measurement tools that assess overlapping components of sleep, which would result in a technical artifact rather than a genuine biological phenomenon. This alternative explanation warrants explicit consideration in the manuscript.
2. Lines 254-257: The observation that not many species are associated with all three sleep characteristics raises concerns about its biological significance. This finding could be influenced by differences in data distributions across the sleep measurements. For instance, as shown in Figure 1b, most sleep quality measures are concentrated at the good-quality end with decent variability, while social jet lag measurements show limited variability at the good-quality end and are skewed towards poor quality. Furthermore, the analyses for social jet lag and chronotype may lack sufficient statistical power, making the results potentially driven by random noise. In contrast, the analysis for sleep quality appears better powered. This discrepancy makes the comparability of significant features across the three sleep characteristics questionable and requires further discussion.
3. In this study, the authors described that alpha diversity was inversely associated with sleep quality. This is somehow an inaccurate description, and it needs to be rephrased. According to the results, alpha diversity was inversely associated with PSQI score in this study, not sleep quality, as a higher score implies lower sleep quality. Nevertheless, this result is consistent with the notion that a higher microbiome diversity is generally more positively related to health.
4. Mediation analysis: The authors' approach to mediation analysis is conceptually flawed. Proper causal mediation analysis requires a pre-specified directed acyclic graph (DAG) and adherence to critical assumptions, including correct model specification. The practice of rotating the roles of exposure, mediator, and outcome to fit different causal mediation models based on statistical significance is fundamentally incorrect. A model should be defined a priori and not selected post hoc based on results. The authors are strongly encouraged to read through VanderWeele's *Explanation in Causal Inference: Methods for Mediation and Interaction* (2015) for proper methodology. Additionally, the study may violate two key assumptions of causal mediation analysis: (1) temporality, i.e., the exposure, mediator, and outcome are measured sequentially; and (2) no unmeasured confounding, as the adjustment for confounders is likely to be insufficient. Finally, addressing high dimensionality upfront in the modeling process, rather than adopting a two-step approach, is critical to avoid overfitting and false positives.
5. The study highlights the role of dietary factors in mediating microbiome changes, but direct effects of diet on sleep characteristics is not discussed in this study. Could you explore more direct relationships between specific dietary patterns (e.g., Mediterranean diet) and sleep quality or chronotype independent of microbiome mediation? At least you should provide it in the discussion.
6. The results presented in Figure 3 lack clarity regarding whether they are multivariable-adjusted. If the models described in the methods were used, additional confounders such as physical activity, sedentary time, dietary quality, total energy intake, alcohol consumption, hypertension, hypercholesterolemia, diabetes, and relevant medications (e.g., sleep aids, melatonin) should also be included. Given the observational nature of the study, adjusting for these variables is essential to strengthen the robustness of the conclusions.
7. How do you reconcile the bidirectional relationship between sleep and the microbiome with the observational nature of the data? Could longitudinal or interventional studies strengthen this aspect? This needs to be at least addressed in the limitation section.
8. The analysis highlights the role of coffee consumption in modulating social jet lag through specific species. There is a paper recently published (doi.org/10.1038/s41564-024-01858-9), showing the strong link between coffee consumption and the species *Lawsonibacter asaccharolyticus*. How is the role of this species in your data? Did it also show a relationship with coffee intake and sleep quality?
9. The variability within genera (e.g., *Blautia*, *Dorea*) is intriguing but raises questions about the deeper relevance of these associations. Including strain-level profiling and functional pathway analyses for the identified species could be interesting for further studies to provide more detailed mechanistic insights.
10. Discuss the broader implications on how the identified microbiome-sleep associations could inform public health strategies or medicine approaches for sleep disorders.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The authors explored the relationship between sleep characteristics, health factors, and the gut microbiome using data from a large-scale prospective population cohort, the Lifelines Dutch Microbiome Project. They performed alpha diversity, beta diversity, and species-level analyses, identifying numerous significant associations. Notably, they conducted mediation analysis to examine the role of the gut microbiome in mediating the effects of coffee intake and social jet lag, uncovering novel findings at the species level. While the research questions are compelling and the use of large-scale microbiome data impressive, the authors must address the concerns outlined below for me to recommend acceptance of the manuscript.

1) The data cannot be found. The authors should disclose the raw sequence data and processed data, preferably including the database, access numbers, and/or website. Without this information, it is impossible to verify the discoveries reported in the study. The authors should clearly organize the processed data for the same 6,941 subjects in the following format:

Feature Table: This table should include raw counts, with rows representing features (ASVs) and columns representing subjects. Row names should be feature IDs, and column names should be subject IDs.

Taxonomic Table: This table should include taxonomic classifications, with rows representing features and columns corresponding to the seven taxonomic ranks: 'Kingdom,' 'Phylum,' 'Class,' 'Order,' 'Family,' 'Genus,' and 'Species.' Row names should be feature IDs.

Metadata: This table should contain variables for phenotypes, disease status, or environmental/dietary factors. Rows should represent subjects, and columns should represent variables, with row names as subject IDs and column names as variable names. The authors should also provide a data dictionary to describe the variables in the metadata in detail.

Phylogenetic Tree: This should be a rooted tree, with tip labels corresponding to feature IDs.

Without this, the discoveries reported in the study cannot be reproduced by others. The phyloseq package (<https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html>) is highly useful for data preparation, though the authors may consider using an alternative comparative software package if preferred. The authors can refer to the following website if they wish. <https://github.com/angelolimeta/Gut-microbiome-immunotherapy>

2) Alpha- and beta-diversity analysis: The authors conducted alpha-diversity and beta-diversity analyses using only few abundance-based indices (Shannon and Bray-Curtis) only. The authors need to survey other widely used phylogenetic indices such as phylogenetic diversity (PD) for alpha diversity and UniFrac distances for beta diversity.

3) Statistical Analysis for Repeated Measurements: Since the data come from the prospective Lifelines Dutch Microbiome Project, there may be repeated measurements for the same subjects. If this is the case, the metadata should include both subject IDs and measurement IDs. Treating repeated measurements as independent subjects can artificially inflate the number of discoveries, leading to numerous spurious findings. The authors should employ appropriate statistical methods, such as mixed-effects models, to account for repeated measurements in their analysis.

For PERMANOVA, 1,000 permutations is too few; it is recommended to use 100,000 permutations. Additionally, PERMANOVA does not account for repeated measurements or covariate adjustments; thus, you can consider mixed-effects model-based methods.

4) Mediation analysis: "employing the mediation package in R" is not at all sufficient. The authors need to disclose the method name and reference. The authors need to disclose how the repeated measurements and covariate adjustments were handled with more specific methodological descriptions.

The authors should provide references for the statement: "Variable selection in the regularized mediation model was performed using a lasso-penalized model, with the optimal lambda for the lasso (L1) penalty determined by minimizing the Bayesian Information Criterion through a grid search."

Furthermore, I recommend performing mediation analysis for both alpha- and beta-diversity indices as well.

Other comments:

A reference needs to be provided for the following statement: "Before conducting any statistical analyses, we applied inverse rank normal transformation to the sleep indices (PSQI score, ESS score, social jet lag, and MSFsc score) to reduce

skewness and improve normality.”

The statement “To correct for multiple testing, we estimated the FDR by conducting 1,000 rounds of permutation” is unclear and requires further clarification.

The authors can also conduct functional (gene content) analyses in addition to the alpha-diversity, beta-diversity, and species analyses.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors thoroughly explained their revisions and efforts, and all comments were addressed satisfactorily.

Reviewer #2

(Remarks to the Author)

Thanks to the authors for their efforts in addressing my previous comments. Most concerns have been largely resolved. However, I have two remaining points regarding the authors' responses:

Point 2:

This issue relates less to non-normality or outliers, and more to the fundamental nature of the variables themselves. The authors' arguments, such as treating them as continuous and applying transformations, do not sufficiently address the concern that the observed associations may be artifacts without clear biological relevance. I still believe that these methodological choices alone cannot rule out the possibility that the findings lack meaningful interpretation.

Point 4:

The authors appear to have rotated the directionality of exposure, mediator, and outcome in their mediation analysis, which is not consistent with the assumptions of classical mediation frameworks. This reorientation also compounds concerns about multiple comparison adjustments, particularly in the context of an already high-dimensional analysis. Furthermore, the use of univariate models as a screening step for multivariable modeling is methodologically problematic. This approach inflates the risk of Type I errors (false positives) due to multiple comparisons and reduces power to detect true associations in a joint model (Type II errors). As a result, the final models may be biased—either overestimating the importance of certain predictors or overlooking key relationships.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

There are several critical issues with the provided datasets, including the feature table, taxonomic table, and phylogenetic tree. Most notably, the feature table does not contain raw count data, contrary to the authors' claim, and the feature IDs are inconsistent across the three datasets. The manuscript states that there are 6,941 subjects, yet the feature table includes 8,355, with no explanation or method provided to extract the 6,941 subjects referenced. Furthermore, without metadata, it is impossible to verify the authors' claims. As a result, the findings presented in the manuscript amount to unsubstantiated assertions. The absence of transparent data reporting is detrimental to scientific integrity. Without proper data verification, I cannot recommend this manuscript for publication.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Thanks for the authors' efforts. I do not have further comments.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

I thank the authors for their clarifications and additional information regarding data access and cohort description. However, the key concerns remain unresolved. The metadata and underlying data needed to independently verify the analyses are still inaccessible, and the criteria for deriving the analysis subset cannot be confirmed.

As reproducibility and data transparency are essential for scientific validation, I am unable to verify the reported results. Therefore, I cannot recommend this manuscript for publication at this stage.

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Response to Reviewers

The Interplay of Sleep Characteristics with Health factors and Gut Microbiome

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The paper is in general clear and well-written. I have, however, some relevant comments and there are few questions and points to be clarified.

Major comments:

Point 1: Indeed, while the authors chose dietary factors (also an intervenable factor) to understand the causal relationship between sleep and the gut microbiome, I still think it's important to address other factors that are highly correlated with sleep traits in mediation analyses, such as gastrointestinal discomfort and mental - depression. With such a large dataset of sleep-microbiome, this is a great and unique opportunity to disentangle the complex relationships among them. Also, for the mediation analyses, the authors concentrated on 137 species, how about the overall microbial composition (beta-diversity, e.g., using principal components).

Response: We thank the reviewer for this thoughtful and helpful suggestion. In addition to dietary factors, we agree that gastrointestinal discomfort and mental health symptoms are important traits closely related to sleep, and that considering them in mediation analyses may further clarify their potential connections with the gut microbiome.

To address this, we extended our mediation analysis framework to include models involving gastrointestinal symptoms (including bloating, abdominal pain, nausea, and diagnosed gastrointestinal disorders such as celiac disease, Crohn's disease, ulcerative colitis, and stomach ulcer) as well as mental health conditions (including depression, generalized anxiety, bipolar disorder, panic disorder, and eating disorder). We tested two pre-defined directions: disease → microbiome → sleep and sleep → microbiome → disease. In these models, we focused specifically on testing whether microbial features act as potential mediators. Unlike diet, which is more clearly established as an environmental exposure to the microbiome, disease can both influence and result from microbiome changes. This makes the directionality more complex and introduces a large number of possible models. To keep the analysis focused and interpretable, we therefore limited our analysis to testing the microbiome as a mediator.

Despite the biological relevance of these pathways and the observed phenotypic associations, we did not detect any statistically significant mediation effects in these models (Supplementary Tables 13–14). This may reflect the limitations of our cross-sectional study design, as well as the relatively low prevalence of diagnosed disease in this population-based cohort, which together reduce the statistical power to detect robust mediation signals in disease-related pathways.

We also appreciate the suggestion to consider overall gut microbial composition in our mediation framework. In the revised analysis, we included both alpha diversity (Shannon index and Phylogenetic

Diversity) and beta diversity (using the first 20 principal coordinates from weighted UniFrac distances) alongside species-level features.

While species-level features remained the primary contributors to mediation effects, including these broader compositional metrics allowed us to more comprehensively assess whether community-level variation may contribute to sleep-related pathways. In the penalized model of the sleep → diet → microbiome direction, we observed that both social jet lag and chronotype exerted indirect effects on phylogenetic diversity via coffee and alcohol consumption, respectively. These updates are now reflected in the revised Methods and Results sections, and the corresponding results are included in Supplementary Tables 8-14.

Method: “A large proportion of the associations we observed between sleep characteristics and other traits were related to dietary patterns, GI discomfort, and mental health conditions. Given that diet is a well-established modulator of the gut microbiome, and GI and mental disorders often co-occur with both sleep disturbances and microbiome alterations, we sought to explore the potential causal relationships between sleep, the microbiome, and either diet or disease. Based on biological plausibility, we pre-specified a set of hypothesized models for mediation analysis. For diet, we tested three plausible pathways: (i) diet → microbiome → sleep, (ii) diet → sleep → microbiome, and (iii) sleep → diet → microbiome. For disease-related traits (GI symptoms and mental health disorders), we tested models where the microbiome may mediate the relationship between disease and sleep: (i) disease → microbiome → sleep and (ii) sleep → microbiome → disease.

Across all models, microbial variables included two types of features: (i) relative abundances of 137 microbial species previously found to be associated with at least one sleep phenotype and (ii) measures of overall microbiome composition. For alpha diversity, we included Shannon index and PD. For beta diversity, we computed the first 20 principal coordinates (PCoAs) based on weighted UniFrac distances. For sleep characteristics, we focused on variables that demonstrated a significant link with species abundance, namely sleep quality, social jet lag, and chronotype. Dietary variables included the Healthy Eating Index (HEI), protein score, alcohol consumption (adjusted for total caloric intake), alternative Mediterranean diet score (aMED), modified Mediterranean diet score (mMED), plant protein score, coffee consumption, total caloric intake, low carbohydrate score, and free sugar intake. The GI phenotypes included celiac disease, Crohn’s disease, ulcerative colitis, stomach ulcer, and general GI discomfort including abdominal pain, bloating, flatulence, burping, and nausea. The mental health phenotypes included bipolar disorder, depression, eating disorder, generalized anxiety, and panic disorder. All microbial variables were adjusted for technical factors (sequencing batch, DNA concentration, and post-QC read count), stool characteristics (Bristol Stool Scale, defecation frequency), and intrinsic factors (age and sex). All other variables were also adjusted for age and sex. Residuals from these adjusted models were used as inputs to the mediation framework to reduce confounding by known covariates.

*As an initial screening step, we conducted univariable mediation analyses using the mediate function from the mediation R package³⁹, systematically testing all relevant combinations of exposures, mediators, and outcomes across the pre-specified model directions (**Table S8–9**). This function estimates the average causal mediation effect and average direct effect based on two linear models: a mediator model (regressing the mediator on the exposure) and an outcome model (regressing the outcome on both the exposure and mediator). For each exposure–mediator–outcome triplet, the mediation effect and its significance were estimated using quasi-Bayesian Monte Carlo simulation with 1,000 draws. These univariable models served as an exploratory filter to identify candidate variables for multivariable analysis. Triplets with a mediation P-value < 0.05 were considered nominally significant, and microbiome variables that appeared in at least one significant triplet were retained for further modeling using a regularized multivariable mediation framework.*

Next, multivariable mediation effects for each direction were estimated using selected microbiome variables using the *regmed* package in R for regularized mediation analysis via penalized structural equation modeling⁴⁰. Variable selection in the regularized mediation model was performed using a lasso-penalized model, with the optimal lambda for the lasso (L1) penalty determined by minimizing the Bayesian Information Criterion through a grid search⁴¹. A non-penalized model incorporating the identified variables was then employed to compute precise effect sizes. For each pathway, the proportion of mediation attributable to the mediator was quantified based on the effect sizes from the non-penalized model. This proportion was determined by dividing the mediated effect (the product of the pathways from X to the mediator and from the mediator to Y) by the total effect (the sum of the mediated effect and any direct effect).”

Results: “Having established a link between sleep and bacterial composition, we sought to determine whether alterations in sleep patterns influence microbial species abundance and diversity measures, or if the reverse is true. To investigate this, we incorporated dietary patterns, which we hypothesized to be more likely to be an exposure than a consequence of the microbial features, into our mediation network analysis. This analysis tested three predefined potential directional relationships: (i) diet → microbiome → sleep, where changes in microbial features potentially cause alterations in sleep characteristics, (ii) diet → sleep → microbiome, and (iii) sleep → diet → microbiome, where changes in microbial features are outcomes of lifestyle modifications.

In the first scenario (**Figure 4a, Table S10**), we observed that most species demonstrated a direct link to either sleep or diet. However, two species, *Clostridia bacterium UC5_1_1E11* and *Clostridia unclassified SGB14844* (both from the unclassified *Clostridia* genus), acted as putative mediators in the diet-to-sleep pathway, suggesting a potential mediation of the effect of coffee consumption on social jet lag. Increased coffee intake was found to boost the abundance of these two species, consequently intensifying the extent of social jet lag. This interesting dynamic between coffee consumption, the two *Clostridia* species, and social jet lag was also observed in the second (**Figure 4b, Table S11**) and third scenarios (**Figure 4c, Table S12**), indicating that fluctuations in social jet lag might impact coffee consumption, or vice versa, thereby altering the abundance of these two *Clostridia* species.

Since these two species appeared in a mediation relationship in all three directions, we could quantify the proportion of mediation based on effect sizes derived from an unpenalized model (**Figure 4d**). By aggregating the effects of *UC5_1_1E11* and *SGB14844*, we discovered that the abundance of these species mediated 10% of the effect of coffee intake on social jet lag. Conversely, social jet lag accounted for only 1.7% of the effect of coffee intake on these species. Notably, coffee intake was found to mediate 59.8% of the effect of social jet lag on these species. These estimates suggest that variation in the abundance of these two *Clostridia* species related to social jet lag may largely reflect differences in coffee consumption.

To further investigate the functional and evolutionary basis of these two *Clostridia* species, we conducted exploratory analyses of their metabolic pathways and phylogenetic relationships. Despite their putative role in mediation, we identified no significant ($FDR < 0.05$) metabolic pathways or phylogenetic conservation of these species’ associations with social jet lag or coffee intake (**Table S13–14**). While their functional roles remain unclear, these species could influence circadian rhythms through unmeasured metabolites or indirect mechanisms, warranting deeper functional profiling in targeted studies.

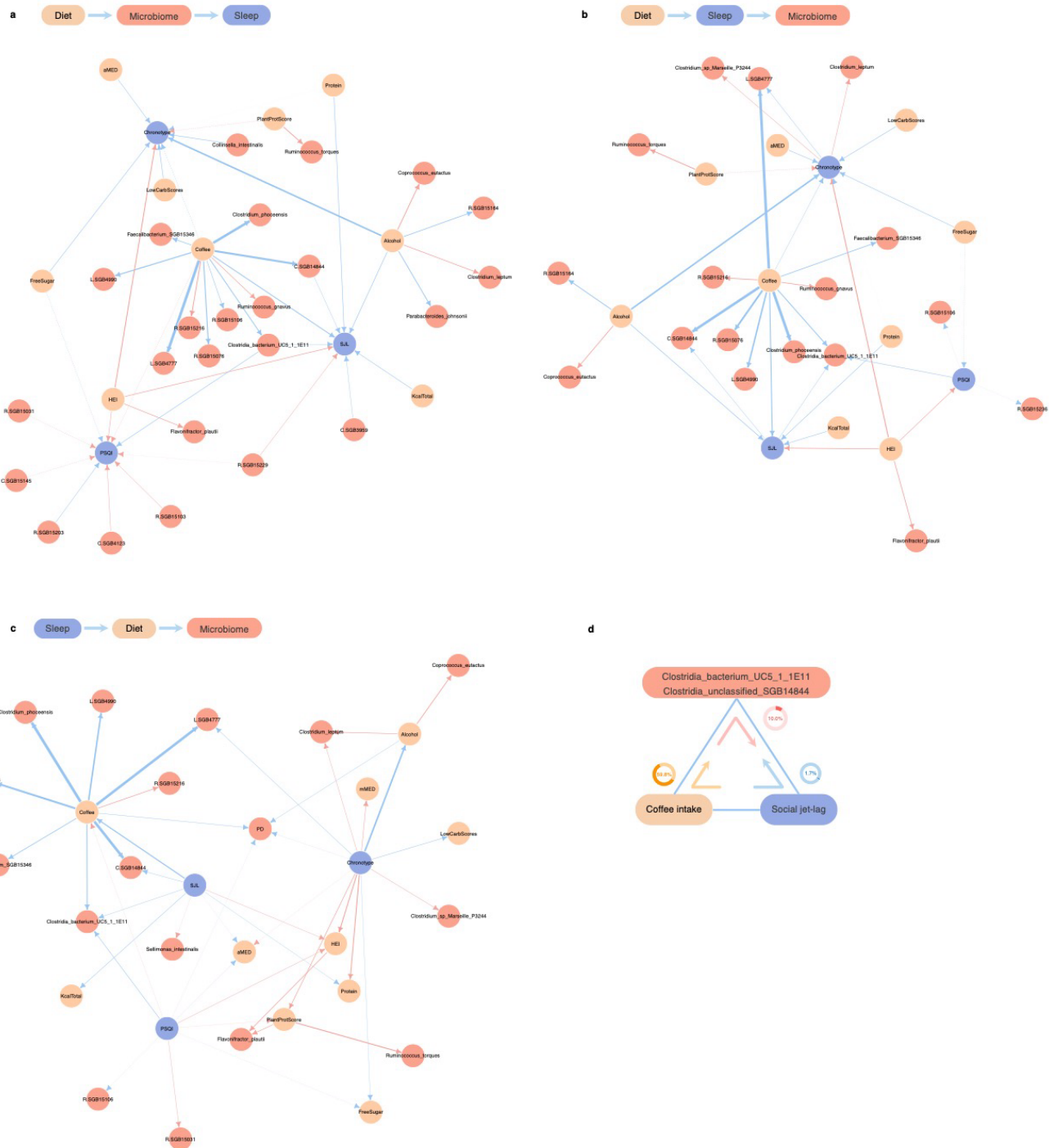
Along the same line, the connections between many microbial features and sleep characteristics were found to be potentially mediated by specific dietary factors (direction III) (**Figure 4c, Table S12**). Similar to the two *Clostridia* species, many species abundances could also be affected by changes in coffee consumption induced by social jet lag. *Lachnospiraceae GGB3570_SGB4777*, *Ruminococcaceae GGB9623_SGB15076*, and *Clostridium phoceensis* showed an increase following coffee intake, while *Ruminococcaceae GGB9699_SGB15216* exhibited a decrease. Additionally, a preference for a later

chronotype was linked with increased alcohol consumption, which in turn led to a reduced abundance of Coprococcus eutactus. Poor sleep quality associated with a lower HEI led to an increased abundance of Flavonifractor plautii. Nevertheless, findings from direction II still revealed that certain species do exhibit a direct connection with sleep patterns, independent of dietary mediation (Figure 4b, Table S11). For example, individuals with a late chronotype displayed elevated levels of Lachnospiraceae GGB3570_SGB4777 and a reduction in Clostridium sp. Marseille P3244 and Clostridium leptum.

To extend the analysis beyond dietary exposures, we also evaluated whether the gut microbiome could serve as a mediator in associations between sleep characteristics and disease phenotypes, focusing on GI symptoms and mental health conditions (Table S15-16). However, we did not identify any microbial features or diversity measures that met the criteria for significant mediation in these models.”

Point 2: And for the data presentation, since the authors have involved three potential directional relationships, it would be desirable to visualize the main findings more clearly in the figure, such as the Figure 4d, which may help the readers to quickly capture several key mediation pathways (what are exposures, mediators and outcomes).

Response: We thank the reviewer for this valuable suggestion. To improve clarity and enhance visualization of the key mediation pathways, we have refined Figure 4 to better illustrate the relationships among exposures, mediators, and outcomes across the three potential directional frameworks.



Point 3: The mediating effect, although significant, remains questionable as to its clinical implications. For example, line 295, “By aggregating the effects of UC5_1_1E11 and SGB14844, we discovered that the abundance of these species mediated 10% of the effect of coffee intake on social jet lag.” Does this represent mainly a direct effect of coffee on social jet lag? How do we interpret the 10% mediation effect in this scenario?

Response: We sincerely thank the reviewer for their insightful comment. First, we would like to clarify the interpretation of the effect size in our mediation analysis. Our finding that two *Clostridia* species mediate approximately 10% of the effect of coffee intake on social jet lag indicates that this portion of the total

effect is mediated indirectly through these microbial species. The remaining 90% of the effect may be attributable to either a direct effect or indirect effects mediated by other factors. Second, we fully agree with the reviewer that the clinical implications of this finding require further investigation. Sleep behavior is complex and influenced by a wide range of physiological and lifestyle factors. A 10% mediation effect is relatively substantial for a multifactorial trait such as social jet lag. Nevertheless, the extent to which social jet lag can be modified through coffee consumption or microbiome-targeted strategies requires future clinical trials. We acknowledge this limitation and have now explicitly discussed it in the revised Discussion section:

“In particular, two Clostridia species, UC5_1_1E11 and SGB14844, were consistently involved in the interplay between coffee consumption and social jet lag across multiple mediation scenarios. Together, the abundance of these two species mediated approximately 10% of the association between coffee intake and social jet lag. Although the majority of the effect is likely direct or mediated through other factors, the observed mediation suggests that these species may play a meaningful biological role and may represent potential targets for mitigating the impact of coffee intake on social jet lag.”

Point 4: The PSQI, as the authors mentioned, is a global sleep quality scale that includes different components, some of which actually overlap with other scales (e.g., the ESS and the PSQI-Q7). It would be interesting to look at the associations of gut microbiome and component scores, e.g., sleep duration & efficiency, which would provide a more comprehensive picture of the sleep-microbiome relationships.

Response: We thank the reviewer for this valuable suggestion. Following the reviewer’s suggestion, we performed additional analyses on each of the seven PSQI components individually (Supplementary Table 6). We identified a total of 140 unique microbial species that showed significant associations with at least one PSQI component. The most associations were observed for PSQI-Q1 (Subjective sleep quality; $n = 80$), PSQI-Q7 (Daytime dysfunction; $n = 58$), and PSQI-Q6 (Use of sleep medication; $n = 44$). When comparing these results with those from the global score, we found that the component analyses largely reflected the patterns already observed with the global PSQI score, with very few additional signals identified (Supplementary Figures 4 and 5). We also did not observe any overlap between associations with daytime dysfunction (PSQI-Q7) and the Epworth Sleepiness Scale (ESS), despite both relating to aspects of daytime impairment. We believe this discrepancy arises from both methodological and conceptual differences. The ESS assesses physiological sleep propensity, the likelihood of dozing off in low-stimulation situations, across eight items, yielding a broad and continuous score (0–24). In contrast, PSQI-Q7 consists of only two items addressing subjective difficulties staying awake and maintaining enthusiasm, rated on a 0–3 frequency scale over the past month. Thus, PSQI-Q7 is more reflective of perceived tiredness, motivation, or mood, while the ESS better captures latent hypersomnolence. These differences in psychometric structure, item framing, and time frame likely contribute to the non-overlapping microbiome associations we observed.

Moreover, due to the ordinal and limited nature of the PSQI component scales (0–3 or 0–4), they offer less variability and sensitivity compared to continuous measures like the ESS or the global PSQI score, possibly reducing the power to detect robust associations. We have now added this additional analysis in the Results section, which reads:

“To further explore specific aspects of sleep, we also examined associations between species and individual PSQI component scores. These analyses largely reflected the patterns observed with the global PSQI score, with most signals captured by the global score (Table S6, Figure S4 and S5).”

We also attach Supplementary Figure S5 here for reference; however, due to its large size, Supplementary Figure S4 could not be included—please refer to the supplementary materials for the full figure.

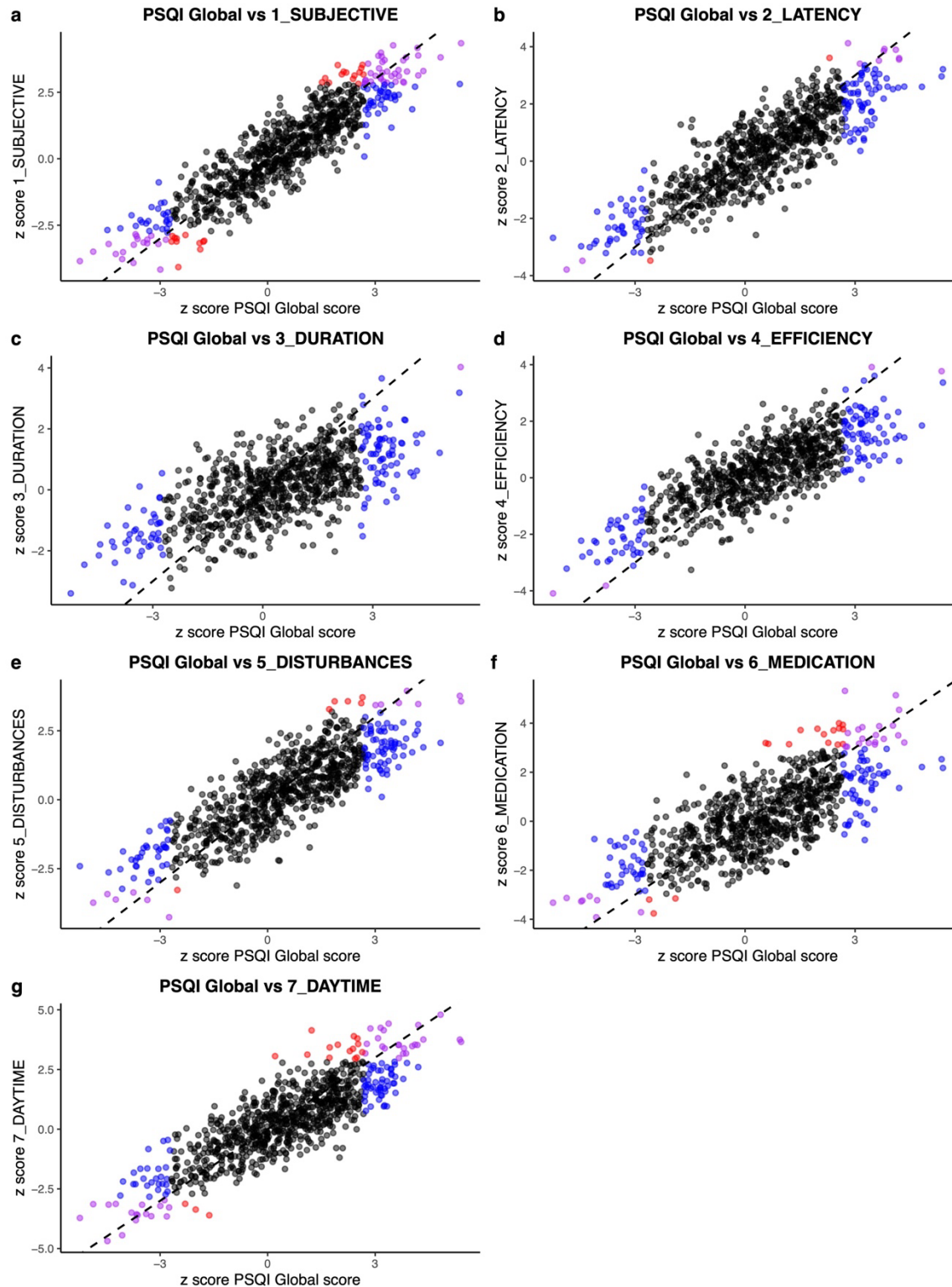


Figure S5: Association between PSQI global score and individual PSQI components across microbial species.

Each point represents a single association between sleep characteristic and microbial species abundance (z-score based). Colors indicate significance: purple = significant in both PSQI global and component association; blue = significant in PSQI global only; red = significant in PSQI component only; black = not significant. The dashed line ($y = x$) represents perfect agreement between global and component scores.

Point 5: In addition, are gut microbial characteristics different across subgroups? E.g., among three chronotypes, as well as those with good and poorer sleep quality

Response:

In our analyses, we treated most sleep indices, including the PSQI global score and midpoint of sleep, as continuous variables. For chronotype, we coded the variable as 0 (Early), 1 (Intermediate), and 2 (Late) when associating with species abundance. This approach was chosen to retain as much information and statistical power as possible. The linear regression model generally reflects the microbial differences across different subgroups. To demonstrate it, we compared the estimated effect size (z-scores) from linear regression models treating the sleep index as continuous versus categorical factors (see Figure below). Overall, we observed a high consistence between two models. It is also not surprising that the linear model using continuous values yielded more significant associations.

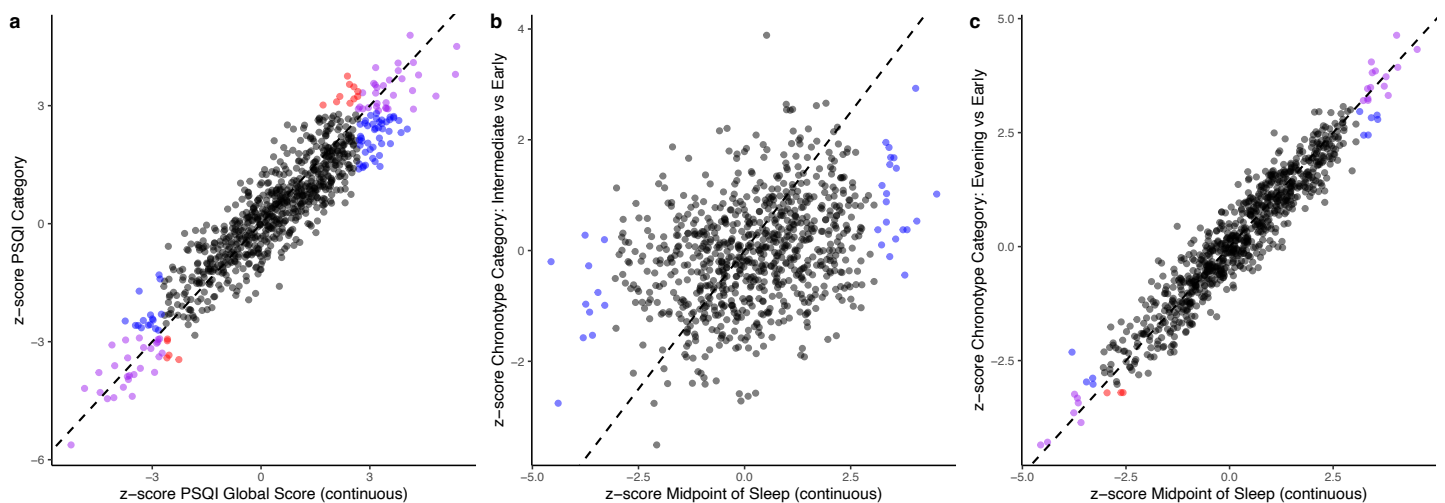


Figure. Comparison of microbial associations between continuous and categorical models

Each panel displays results from two modeling approaches assessing microbial associations with sleep-related traits. Each point represents a microbial species. Blue points indicate species significantly associated with the continuous variable only. Red points represent species differing significantly across categorized subgroups only. Purple points mark species significant in both models.

Based on these findings, we continued to use continuous variables in the main analyses, as they retain more information across the full range of the sleep traits and offer better sensitivity for detecting associations.

Other minor comments:

Point 6: In the abstract, line 15-16, “We found that alpha diversity was inversely associated with sleep quality, chronotype, and social jet lag,..”, either rephrased as “was associated with” or specify: “was inversely associated with poorer sleep quality, earlier chronotype..”.

Response: We thank the reviewer for the helpful suggestion. We have revised the sentence to clarify the nature of the inverse associations. The sentence now reads: “*We found that lower alpha diversity was associated with poorer sleep quality, later chronotype, and greater social jet lag.*”

Point 7: In methods, line 135-136, “To focus on the most relevant species, we filtered out species with a mean relative abundance present in fewer than 100 participants, leaving 813 species for further analysis.” – it is unclear, whether the authors filtered out species occurring in fewer than 100 participants (? %), or did they filter out species below a certain average relative abundance? Or both?

Response: We thank the reviewer for pointing this out. We apologize for the confusion. We filtered out species that were present in fewer than 100 participants (approximately 1.4% of the cohort), regardless of their relative abundance. We have now clarified this sentence in the Methods section as: *“To focus on the most relevant species, we filtered out species that were detected in fewer than 100 participants (approximately 1.4% of the cohort), leaving 813 species for further analysis.”*

Point 8: In discussion, line 379-380, “When considering diet as an exposure, we identified some connections that suggest a direct causal link between sleep patterns and variations in species abundance”, do the authors mean consider diet as a mediator?

Response: We thank the reviewer for this important comment. We agree that the original wording was unclear. In this analysis, we considered diet as the exposure and sleep characteristics as potential mediators between diet and microbiome species abundance. We have revised the sentence in the Discussion to: *“When considering diet as the exposure and sleep characteristics as potential mediators, we identified some connections that suggest a direct link between sleep patterns and variations in species abundance.”*

Reviewer #2:

The manuscript presents a comprehensive analysis of the bidirectional relationship between sleep characteristics and gut microbiome composition in a large-scale Dutch cohort. The integration of various statistical methods, metagenomic sequencing that ensures detailed taxonomic profiling at the species level, improving our understanding of the complex interplay between sleep and gut microbiome composition. The identification of specific bacterial species associated with distinct sleep parameters is particularly interesting. However, some areas, such as the analysis methods and interpretation of results, require further clarification and additional analysis to strengthen the conclusions. Our specific comments are:

Point 1: Lines 241-243: The authors report a “consistent directional trend” across species associated with positive sleep characteristics. This interpretation as a biologically meaningful finding is questionable. Instead, the trend could be due to the use of measurement tools that assess overlapping components of sleep, which would result in a technical artifact rather than a genuine biological phenomenon. This alternative explanation warrants explicit consideration in the manuscript.

Response: We thank the reviewer for this valuable point. Indeed, the consistent directional trend we observed across species may be partly due to correlations among the sleep measures. To address this, we have now explicitly pointed it out and revised the corresponding part of the Results section: *“Among the 137 species identified, we observed a consistent directional trend, whereby a species associated with a positive sleep characteristic (e.g., good sleep quality) tended to also associate positively with other favorable sleep characteristics (e.g., reduced social jet lag). This may suggest a shared biological basis underlying different aspects of sleep, although some of the observed consistency could also reflect overlap among the sleep measures.”*

Point 2: Lines 254-257: The observation that not many species are associated with all three sleep characteristics raises concerns about its biological significance. This finding could be influenced by differences in data distributions across the sleep measurements. For instance, as shown in Figure 1b, most sleep quality measures are concentrated at the good-quality end with decent variability, while social jet lag measurements show limited variability at the good-quality end and are skewed towards poor quality. Furthermore, the analyses for social jet lag and chronotype may lack sufficient statistical power, making the results potentially driven by random noise. In contrast, the analysis for sleep quality appears better powered. This discrepancy makes the comparability of significant features across the three sleep characteristics questionable and requires further discussion.

Response: We thank the reviewer for this thoughtful comment. While we agree that the original distributions of sleep traits differ (Figure 1b), we note that in the association analyses, sleep quality (PSQI) and social jet lag were treated as continuous variables and normalized using inverse rank transformation. This approach minimizes the impact of non-normal distributions and helps maintain comparable statistical power across these traits. Chronotype, on the other hand, was categorized into three ordinal groups (early, intermediate, late), coded as 0, 1, and 2. We acknowledge that the limited number of categories for chronotype could introduce some granularity and potentially reduce power compared to fully continuous traits. We have clarified this point in the Results section while keeping the main text concise to maintain readability: *“Most of the sleep–species associations we identified were uniquely linked to a specific sleep aspect: 101 species were exclusively associated with sleep quality, 8 with social jet lag, and 7 with chronotype. The larger number of associations we observed for sleep quality might not only indicate stronger biological connections, it could also partly result from greater variability in the sleep quality data compared to other sleep measures.”*

Point 3: In this study, the authors described that alpha diversity was inversely associated with sleep quality. This is somehow an inaccurate description, and it needs to be rephrased. According to the results, alpha diversity was inversely associated with PSQI score in this study, not sleep quality, as a higher score implies lower sleep quality. Nevertheless, this result is consistent with the notion that a higher microbiome diversity is generally more positively related to health.

Response: We thank the reviewer for this important suggestion. To improve precision, we have revised the wording in both the Abstract and the Results section. In the Abstract, we now state: *“We found that lower alpha diversity was associated with poorer sleep quality, later chronotype, and greater social jet lag.”* In the Results section, we specify: *“Alpha diversity, as measured by the Shannon index, was significantly associated with PSQI score (Beta = -0.015, P-value = 0.017),”* thereby clearly indicating that the association was with the PSQI score, where higher values reflect poorer sleep quality.

Point 4: Mediation analysis: The authors' approach to mediation analysis is conceptually flawed. Proper causal mediation analysis requires a pre-specified directed acyclic graph (DAG) and adherence to critical assumptions, including correct model specification. The practice of rotating the roles of exposure, mediator, and outcome to fit different causal mediation models based on statistical significance is fundamentally incorrect. A model should be defined a priori and not selected post hoc based on results. The authors are strongly encouraged to read through VanderWeele's *Explanation in Causal Inference: Methods for Mediation and Interaction* (2015) for proper methodology. Additionally, the study may violate two key assumptions of causal mediation analysis: (1) temporality, i.e., the exposure, mediator, and outcome are measured sequentially; and (2) no unmeasured confounding, as the adjustment for

confounders is likely to be insufficient. Finally, addressing high dimensionality upfront in the modeling process, rather than adopting a two-step approach, is critical to avoid overfitting and false positives.

Response: We appreciate the reviewer's careful critique and fully agree that causal mediation analysis requires strong assumptions, including a clearly specified causal structure, temporally ordered variables, and appropriate adjustment for confounding. We would like to clarify that our study employs mediation analysis in an exploratory, hypothesis-generating context, rather than to make formal causal claims.

To avoid post hoc model selection, all directional models were pre-specified based on biological plausibility. Specifically, we defined three directional hypotheses involving diet (diet → microbiome → sleep, diet → sleep → microbiome, sleep → diet → microbiome) and two involving disease (disease → microbiome → sleep, sleep → microbiome → disease). The diet models were selected based on the recognition that diet is generally considered upstream of the microbiome due to its well-established role as a primary modulator of microbial composition. For the disease-related models, we explored these directions to assess the potential for the gut microbiome to act as a mediator. If such mediation exists, it could point toward microbiome-targeted strategies for disease prevention or intervention. These five directions were tested consistently across all combinations, independent of statistical significance. We did not rotate the roles of exposure, mediator, and outcome based on results. This structure was established prior to analysis to ensure clarity and interpretability.

We acknowledge that our cross-sectional design limits our ability to establish temporality and that unmeasured confounding remains a possibility despite extensive covariate adjustment. We have now made these limitations more explicit in the revised manuscript and have taken care to frame our findings as exploratory and hypothesis-generating. We hope our results will inform future studies that can more directly assess causal relationships using longitudinal or interventional designs.

With respect to high dimensionality, we recognize the importance of minimizing overfitting and controlling false positives. In our approach, the univariable mediation analysis served as a screening step to reduce the dimensionality of microbial features prior to multivariable modeling. Including all ~200 microbial variables in a single penalized model can result in excessive shrinkage and unreliable effect estimation, making pre-selection a necessary compromise. The final effect estimates were obtained solely from the penalized multivariable model, which applies an L1 (lasso) penalty to enable robust variable selection and effect estimation. We believe this strategy improves model stability and interpretability while helping to mitigate the risk of overfitting in a high-dimensional setting.

We have revised the manuscript accordingly to clarify the exploratory nature of our mediation analysis, the assumptions involved, and the rationale behind our modeling strategy. These revisions are now reflected in:

Method: "A large proportion of the associations we observed between sleep characteristics and other traits were related to dietary patterns, GI discomfort, and mental health conditions. Given that diet is a well-established modulator of the gut microbiome, and GI and mental disorders often co-occur with both sleep disturbances and microbiome alterations, we sought to explore the potential causal relationships between sleep, the microbiome, and either diet or disease. Based on biological plausibility, we pre-specified a set of hypothesized models for mediation analysis. For diet, we tested three plausible pathways: (i) diet → microbiome → sleep, (ii) diet → sleep → microbiome, and (iii) sleep → diet → microbiome. For disease-related traits (GI symptoms and mental health disorders), we tested models where the microbiome may mediate the relationship between disease and sleep: (i) disease → microbiome → sleep and (ii) sleep → microbiome → disease.

Across all models, microbial variables included two types of features: (i) relative abundances of 137 microbial species previously found to be associated with at least one sleep phenotype and (ii) measures

of overall microbiome composition. For alpha diversity, we included Shannon index and PD. For beta diversity, we computed the first 20 principal coordinates (PCoAs) based on weighted UniFrac distances. For sleep characteristics, we focused on variables that demonstrated a significant link with species abundance, namely sleep quality, social jet lag, and chronotype. Dietary variables included the Healthy Eating Index (HEI), protein score, alcohol consumption (adjusted for total caloric intake), alternative Mediterranean diet score (aMED), modified Mediterranean diet score (mMED), plant protein score, coffee consumption, total caloric intake, low carbohydrate score, and free sugar intake. The GI phenotypes included celiac disease, Crohn's disease, ulcerative colitis, stomach ulcer, and general GI discomfort including abdominal pain, bloating, flatulence, burping, and nausea. The mental health phenotypes included bipolar disorder, depression, eating disorder, generalized anxiety, and panic disorder. All microbial variables were adjusted for technical factors (sequencing batch, DNA concentration, and post-QC read count), stool characteristics (Bristol Stool Scale, defecation frequency), and intrinsic factors (age and sex). All other variables were also adjusted for age and sex. Residuals from these adjusted models were used as inputs to the mediation framework to reduce confounding by known covariates.

As an initial screening step, we conducted univariable mediation analyses using the *mediate* function from the mediation R package³⁹, systematically testing all relevant combinations of exposures, mediators, and outcomes across the pre-specified model directions (**Table S8–9**). This function estimates the average causal mediation effect and average direct effect based on two linear models: a mediator model (regressing the mediator on the exposure) and an outcome model (regressing the outcome on both the exposure and mediator). For each exposure–mediator–outcome triplet, the mediation effect and its significance were estimated using quasi-Bayesian Monte Carlo simulation with 1,000 draws. These univariable models served as an exploratory filter to identify candidate variables for multivariable analysis. Triplets with a mediation P-value < 0.05 were considered nominally significant, and microbiome variables that appeared in at least one significant triplet were retained for further modeling using a regularized multivariable mediation framework.

Next, multivariable mediation effects for each direction were estimated using selected microbiome variables using the *regmed* package in R for regularized mediation analysis via penalized structural equation modeling⁴⁰. Variable selection in the regularized mediation model was performed using a lasso-penalized model, with the optimal lambda for the lasso (L1) penalty determined by minimizing the Bayesian Information Criterion through a grid search⁴¹. A non-penalized model incorporating the identified variables was then employed to compute precise effect sizes. For each pathway, the proportion of mediation attributable to the mediator was quantified based on the effect sizes from the non-penalized model. This proportion was determined by dividing the mediated effect (the product of the pathways from X to the mediator and from the mediator to Y) by the total effect (the sum of the mediated effect and any direct effect)."

Results: "Having established a link between sleep and bacterial composition, we sought to determine whether alterations in sleep patterns influence microbial species abundance and diversity measures, or if the reverse is true. To investigate this, we incorporated dietary patterns, which we hypothesized to be more likely to be an exposure than a consequence of the microbial features, into our mediation network analysis. This analysis tested three predefined potential directional relationships: (i) diet → microbiome → sleep, where changes in microbial features potentially cause alterations in sleep characteristics, (ii) diet → sleep → microbiome, and (iii) sleep → diet → microbiome, where changes in microbial features are outcomes of lifestyle modifications.

In the first scenario (**Figure 4a, Table S10**), we observed that most species demonstrated a direct link to either sleep or diet. However, two species, *Clostridia bacterium UC5_1_1E11* and *Clostridia unclassified SGB14844* (both from the unclassified *Clostridia* genus), acted as putative mediators in the diet-to-sleep

pathway, suggesting a potential mediation of the effect of coffee consumption on social jet lag. Increased coffee intake was found to boost the abundance of these two species, consequently intensifying the extent of social jet lag. This interesting dynamic between coffee consumption, the two *Clostridia* species, and social jet lag was also observed in the second (**Figure 4b, Table S11**) and third scenarios (**Figure 4c, Table S12**), indicating that fluctuations in social jet lag might impact coffee consumption, or vice versa, thereby altering the abundance of these two *Clostridia* species.

Since these two species appeared in a mediation relationship in all three directions, we could quantify the proportion of mediation based on effect sizes derived from an unpenalized model (**Figure 4d**). By aggregating the effects of UC5_1_1E11 and SGB14844, we discovered that the abundance of these species mediated 10% of the effect of coffee intake on social jet lag. Conversely, social jet lag accounted for only 1.7% of the effect of coffee intake on these species. Notably, coffee intake was found to mediate 59.8% of the effect of social jet lag on these species. These estimates suggest that variation in the abundance of these two *Clostridia* species related to social jet lag may largely reflect differences in coffee consumption.

To further investigate the functional and evolutionary basis of these two *Clostridia* species, we conducted exploratory analyses of their metabolic pathways and phylogenetic relationships. Despite their putative role in mediation, we identified no significant ($FDR < 0.05$) metabolic pathways or phylogenetic conservation of these species' associations with social jet lag or coffee intake (**Table S13–14**). While their functional roles remain unclear, these species could influence circadian rhythms through unmeasured metabolites or indirect mechanisms, warranting deeper functional profiling in targeted studies.

Along the same line, the connections between many microbial features and sleep characteristics were found to be potentially mediated by specific dietary factors (direction III) (**Figure 4c, Table S12**). Similar to the two *Clostridia* species, many species abundances could also be affected by changes in coffee consumption induced by social jet lag. *Lachnospiraceae* GGB3570_SGB4777, *Ruminococcaceae* GGB9623_SGB15076, and *Clostridium phoceensis* showed an increase following coffee intake, while *Ruminococcaceae* GGB9699_SGB15216 exhibited a decrease. Additionally, a preference for a later chronotype was linked with increased alcohol consumption, which in turn led to a reduced abundance of *Coprococcus eutactus*. Poor sleep quality associated with a lower HEI led to an increased abundance of *Flavonifractor plautii*. Nevertheless, findings from direction II still revealed that certain species do exhibit a direct connection with sleep patterns, independent of dietary mediation (**Figure 4b, Table S11**). For example, individuals with a late chronotype displayed elevated levels of *Lachnospiraceae* GGB3570_SGB4777 and a reduction in *Clostridium* sp. Marseille P3244 and *Clostridium leptum*.

To extend the analysis beyond dietary exposures, we also evaluated whether the gut microbiome could serve as a mediator in associations between sleep characteristics and disease phenotypes, focusing on GI symptoms and mental health conditions (**Table S15–16**). However, we did not identify any microbial features or diversity measures that met the criteria for significant mediation in these models.”

Discussion: “Another compelling area for future research involves uncovering the true causal links between sleep-related disorders, sleep patterns, and the gut microbiome through intervention or experimental studies. However, this topic was beyond the scope of our current study. Given the cross-sectional nature of our data, establishing definitive directionality is challenging because any of the factors could be hypothesized as exposures, mediators, or outcomes, resulting in six different hypothetical scenarios for analysis. While dealing with such complexity using cross-sectional data is challenging, exploring these aspects through future longitudinal studies could help deepen our understanding of these complex relationships.

As mentioned, one of the major limitations of our study is its cross-sectional design, which prevents us from establishing the temporal order of events. This is particularly relevant for mediation analysis, where

a clear sequence of exposure, mediator, and outcome is required to support causal interpretation. In addition, although we adjusted for a broad set of known covariates, we cannot exclude the possibility of unmeasured confounding. Therefore, the mediation results should be viewed as exploratory and interpreted with caution. Moreover, the reliance on self-reported data for both sleep patterns and dietary habits introduces potential reporting bias. Although self-reporting is valuable for gathering data in large-scale studies, it is prone to inaccuracies and personal biases. Objective measurements, such as actigraphy for sleep and detailed diet logs or biomarkers for dietary intake, would enhance the validity of results.”

Point 5: The study highlights the role of dietary factors in mediating microbiome changes, but direct effects of diet on sleep characteristics is not discussed in this study. Could you explore more direct relationships between specific dietary patterns (e.g., Mediterranean diet) and sleep quality or chronotype independent of microbiome mediation? At least you should provide it in the discussion.

Response: We thank the reviewer for the helpful suggestion. Associations of sleep characteristics and dietary patterns are presented in Figure 2. In the result section we wrote: *“Our results showed that individuals with optimal health status exhibited more favorable sleep characteristics. Conversely, suboptimal sleep—defined by lower sleep quality, increased daytime sleepiness, greater social jet lag, and a tendency toward a late chronotype—was linked to a spectrum of health issues, notably psychological and GI disorders. Regarding dietary habits, a healthy diet, as indicated by high HEI scores and increased intake of polysaccharides and proteins, was associated with better sleep metrics. In contrast, diets characterized by adherence to a Western pattern and higher intake of maltose were associated with poorer sleep parameters. Notably, alcohol consumption was linked to a later chronotype ($P\text{-value} = 1.38 \times 10^{-40}$), whereas higher total caloric intake was related to increased daytime sleepiness ($P\text{-value} = 4.47 \times 10^{-25}$).”*

We have now also added a paragraph to the Discussion to highlight the strong link between diet and sleep observed in our current analyses. The added text reads: *“By incorporating dietary factors, we were able to conduct a comprehensive mediation analysis. Diet showed strong direct associations with sleep characteristics; for example, a higher Healthy Eating Index (HEI) was directly linked to better sleep quality, earlier chronotype, and reduced social jet lag; coffee and alcohol consumption were linked to chronotype and social jet lag; and higher alternative Mediterranean diet scores (aMED) and low-carbohydrate diet scores were linked to chronotype. All these associations were observed in both directions.”*

Point 6: The results presented in Figure 3 lack clarity regarding whether they are multivariable-adjusted. If the models described in the methods were used, additional confounders such as physical activity, sedentary time, dietary quality, total energy intake, alcohol consumption, hypertension, hypercholesterolemia, diabetes, and relevant medications (e.g., sleep aids, melatonin) should also be included. Given the observational nature of the study, adjusting for these variables is essential to strengthen the robustness of the conclusions.

Response:

We thank the reviewer for underscoring the importance of robust confounding control. In the revised manuscript, we have updated Figure 3 caption to explicitly indicate that the results are based on multivariable-adjusted models, accounting for age, sex, BMI, batch, DNA concentration, Bristol Stool Scale, and defecation frequency.

We have also conducted supplementary analyses adjusting for the additional confounders suggested. Specifically, we included physical activity, alcohol consumption, metabolic diseases (hypertension,

hypercholesterolemia, and diabetes), alcohol consumption, dietary quality (Healthy Eating Index [HEI]), total energy intake, and use of sleep-aid medications, in addition to GI symptoms and mental diseases. The results of these extended adjustments are presented in the updated Supplementary Figure S6. Adjusting for each covariate individually did not substantially alter the observed associations, indicating that our findings are robust to these potential confounders. Adjustment for sleep-aid medication slightly changed the association with PSQI scores. This is likely because the global PSQI score already incorporates information on sleep medication use (specifically through component 6), and thus adjusting for medication use separately could introduce overadjustment. In addition, we examined a fully adjusted model incorporating all covariates simultaneously. Although the strength of some associations was slightly attenuated due to a reduction in sample size from missing data, the overall directionality and consistency of the findings were preserved. These results further support the robustness of our conclusions. We have updated the Methods and Results sections and Supplementary Figure S6 to document these analyses.

Method: “To account for the potential confounding effects of various factors on the gut microbiome, we included the following factors as covariates when conducting any analyses related to the microbial features: technical parameters (metagenomic sequencing batch number, DNA concentration (ng/μl), and number of post-quality control reads), intrinsic factors (age, sex, and BMI), and stool characteristics (frequency and consistency measured by Bristol Stool Scale).”

To assess the robustness of species abundance associations with sleep characteristics, we performed a series of sensitivity analyses, adjusting individually for additional potential confounders, including metabolic diseases (hypertension, hypercholesterolemia, and diabetes), GI diseases, mental health conditions, physical activity, alcohol intake, and sleep medication use. We also examined a fully adjusted model incorporating all covariates simultaneously.”

*Result: “In our previous analysis, we had observed strong links between sleep characteristics and mental or GI diseases. To assess whether these or other health and lifestyle factors conditions were confounding our results, we conducted a sensitivity analysis incorporating additional covariates, including mental disease status, GI disease status, metabolic diseases (hypertension, hypercholesterolemia, diabetes), physical activity, alcohol intake, and use of sleep medications in our model (**Figure S6**). The consistent distribution of significance and direction of effect, even after accounting for these covariates individually and jointly, indicates that the associations between sleep characteristics and microbial species are robust and not driven by these potential confounders.”*

Point 7: How do you reconcile the bidirectional relationship between sleep and the microbiome with the observational nature of the data? Could longitudinal or interventional studies strengthen this aspect? This needs to be at least addressed in the limitation section.

Response:

We thank the reviewer for the helpful comment. We have emphasized the limitations of our cross-sectional design in the Discussion section: *“Another compelling area for future research involves uncovering the true causal links between sleep-related disorders, sleep patterns, and the gut microbiome through intervention or experimental studies. However, this topic was beyond the scope of our current study. Given the cross-sectional nature of our data, establishing definitive directionality is challenging because any of the factors could be hypothesized as exposures, mediators, or outcomes, resulting in six different hypothetical scenarios for analysis. While dealing with such complexity using cross-sectional data is challenging, exploring these aspects through future longitudinal studies could help deepen our understanding of these complex relationships.*

As mentioned, one of the major limitations of our study is its cross-sectional design, which prevents us from establishing the temporal order of events. This is particularly relevant for mediation analysis, where a clear sequence of exposure, mediator, and outcome is required to support causal interpretation. In addition, although we adjusted for a broad set of known covariates, we cannot exclude the possibility of unmeasured confounding. Therefore, the mediation results should be viewed as exploratory and interpreted with caution. Moreover, the reliance on self-reported data for both sleep patterns and dietary habits introduces potential reporting bias. Although self-reporting is valuable for gathering data in large-scale studies, it is prone to inaccuracies and personal biases. Objective measurements, such as actigraphy for sleep and detailed diet logs or biomarkers for dietary intake, would enhance the validity of results.”

Point 8: The analysis highlights the role of coffee consumption in modulating social jet lag through specific species. There is a paper recently published (doi.org/10.1038/s41564-024-01858-9), showing the strong link between coffee consumption and the species *Lawsonibacter asaccharolyticus*. How is the role of this species in your data? Did it also show a relationship with coffee intake and sleep quality?

Response: Thank you for bringing this interesting recent finding to our attention. Indeed, we observe a strong association between this particular species and both coffee intake and chronotype in our data. However, in our study, this species is annotated as *Clostridium phoceensis* rather than *Lawsonibacter asaccharolyticus*. As noted in the original study, “However, due to the majority voting rule, currently the SGB ID 15154 when profiled by MetaPhlAn 4 reports the taxonomic label *Clostridium phoceensis*. This will be corrected in future database releases of bioBakery 4.”

To ensure consistency with the version of MetaPhlAn and the ChocoPhlAn database used in our profiling, we will retain the current taxonomic label (*Clostridium phoceensis*). However, we have clarified in the Discussion that this species corresponds to *Lawsonibacter asaccharolyticus* as reported in the recent study, to avoid confusion and to highlight that both studies identify the same species as being associated with coffee intake. The revised text reads : “Our observation of a strong association between coffee intake and *Clostridium phoceensis* aligns with a recent study that reported a link between coffee consumption and *Lawsonibacter asaccharolyticus*. As noted in that work, this species corresponds to SGB15154, which is currently annotated as *Clostridium phoceensis* in the version of the database used for our profiling. These consistent findings further support the robustness of coffee–microbiome relationships across independent cohorts and profiling pipelines.”

Point 9: The variability within genera (e.g., *Blautia*, *Dorea*) is intriguing but raises questions about the deeper relevance of these associations. Including strain-level profiling and functional pathway analyses for the identified species could be interesting for further studies to provide more detailed mechanistic insights.

Response: We thank the reviewer for this thoughtful suggestion. To explore the potential for deeper mechanistic insights, we performed additional species-resolved pathway and phylogenetic analyses focused on *Clostridia bacterium UC5_1_1E11* and *Clostridia sp. SGB14844*, which emerged as putative mediators in the relationship between coffee intake and social jet lag. Using HUMAnN 4.0 and MetaCyc pathway annotations, we profiled within-species functional variation, but did not identify specific pathways strongly associated with sleep or dietary traits. We also applied phylogenetic generalized linear mixed models (PGLMMs) using the Anpan R package to assess whether phylogenetic relatedness could explain the observed associations, but found no strong phylogenetic signal. These negative results may

reflect limited statistical power due to the relatively low prevalence and abundance of these species in the cohort.

Nonetheless, we agree that incorporating strain-level and functional profiling is a promising direction for uncovering mechanistic links in microbiome–host interactions. We have now included these exploratory analyses in the revised manuscript (Results and Methods), with full results reported in Supplementary Tables 13 and 14.

Method: “**Functional and phylogenetic profiling of *Clostridia* species**

*To further explore *Clostridia* bacterium UC5_1_1E11 and *Clostridia* sp. SGB14844, we performed species-resolved pathway and phylogenetic analyses. For functional profiling, we used HUMAnN 4.0 to quantify MetaCyc pathway abundances within each species. A total of 106 pathways were detected across the two species (48 in SGB14844 and 58 in SGB14895). Associations between CLR-transformed pathway abundances and phenotypes (coffee intake and social jet lag) were tested using linear models adjusted for age, sex, BMI, DNA concentration, sequencing batch, post-QC read count, Bristol Stool Scale, and defecation frequency. Analyses were performed separately per species among individuals with complete data ($n = 630$ for SGB14844; $n = 77$ for SGB14895).*

To evaluate whether phylogenetic relatedness among strains could help explain phenotype associations, we reconstructed strain-level phylogenies using StrainPhlAn with minimum marker detection thresholds set to 10 across samples. We then fit phylogenetic generalized linear mixed models using the anpan R package⁴², modeling phenotypes as a function of covariates and a phylogenetic random effect under a Brownian motion assumption. Model fit was assessed by comparing the expected log predictive density (ELPD) of each phylogenetic model to a null model without the phylogenetic term, using Pareto-smoothed leave-one-out cross-validation. A phylogenetic signal was considered statistically supported if the ELPD difference (Δ ELPD) between the null and phylogenetic model was less than -4 and the 95% confidence interval (Δ ELPD ± 2 standard errors) did not include zero.”

Result: “*To further investigate the functional and evolutionary basis of these two *Clostridia* species, we conducted exploratory analyses of their metabolic pathways and phylogenetic relationships. Despite their putative role in mediation, we identified no significant ($FDR < 0.05$) metabolic pathways or phylogenetic conservation of these species’ associations with social jet lag or coffee intake (Table S13–14). While their functional roles remain unclear, these species could influence circadian rhythms through unmeasured metabolites or indirect mechanisms, warranting deeper functional profiling in targeted studies.*”

Discussion: “*In particular, two *Clostridia* species, UC5_1_1E11 and SGB14844, were consistently involved in the interplay between coffee consumption and social jet lag across multiple predefined mediation scenarios. Together, the abundance of these two species mediated approximately 10% of the association between coffee intake and social jet lag. Although the majority of the effect is likely direct, or mediated through other factors, the observed mediation suggests that these species may play a meaningful biological role and may represent potential targets for mitigating the impact of coffee intake on social jet lag. Although these species remain poorly characterized, their membership in class *Clostridia*, which includes many species known to influence host metabolism⁵³ and neuroactive compound production⁵⁴, points to plausible mechanisms involving microbial metabolites affecting sleep regulation. To further explore their potential roles, we examined the pathway-level functional profiles and phylogenetic patterns of these two species. However, we did not identify any specific metabolic pathways or phylogenetic signals that could explain their associations with coffee intake or sleep-related traits. These null findings may reflect the relatively low prevalence and abundance of these species in the population, limiting the statistical power to detect subtle biological effects. Nonetheless, their consistent appearance in directional models highlights them as intriguing candidates for future mechanistic investigation.*”

Point 10: Discuss the broader implications on how the identified microbiome-sleep associations could inform public health strategies or medicine approaches for sleep disorders.

Response: We thank the reviewer for the valuable suggestion. We have added sentences in the Discussion to highlight the broader public health implications of our findings. The added text now reads: *“Furthermore, we offer insight into the complex interconnection between diet, sleep, and the microbiome, highlighting potential avenues for public health strategies. For instance, dietary interventions could be a promising strategy for managing sleep disorders and modulating gut health (e.g., reducing coffee/alcohol intake to modulate Clostridia species linked to social jet lag). Similarly, microbiome-targeted interventions, such as probiotics or prebiotics, might mitigate circadian misalignment in shift workers or individuals with late chronotypes. These approaches could complement existing behavioral therapies for sleep disorders, though rigorous clinical trials are needed to evaluate efficacy.”*

Reviewer #3

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

The authors explored the relationship between sleep characteristics, health factors, and the gut microbiome using data from a large-scale prospective population cohort, the Lifelines Dutch Microbiome Project. They performed alpha diversity, beta diversity, and species-level analyses, identifying numerous significant associations. Notably, they conducted mediation analysis to examine the role of the gut microbiome in mediating the effects of coffee intake and social jet lag, uncovering novel findings at the species level. While the research questions are compelling and the use of large-scale microbiome data impressive, the authors must address the concerns outlined below for me to recommend acceptance of the manuscript.

Major Comments:

Point 1: The data cannot be found. The authors should disclose the raw sequence data and processed data, preferably including the database, access numbers, and/or website. Without this information, it is impossible to verify the discoveries reported in the study. The authors should clearly organize the processed data for the same 6,941 subjects in the following format:

1. **Feature Table:** This table should include raw counts, with rows representing features (ASVs) and columns representing subjects. Row names should be feature IDs, and column names should be subject IDs.
2. **Taxonomic Table:** This table should include taxonomic classifications, with rows representing features and columns corresponding to the seven taxonomic ranks: 'Kingdom,' 'Phylum,' 'Class,' 'Order,' 'Family,' 'Genus,' and 'Species.' Row names should be feature IDs.
3. **Metadata:** This table should contain variables for phenotypes, disease status, or environmental/dietary factors. Rows should represent subjects, and columns should represent variables, with row names as subject IDs and column names as variable names. The authors should also provide a data dictionary to describe the variables in the metadata in detail.

4. Phylogenetic Tree: This should be a rooted tree, with tip labels corresponding to feature IDs.

Without this, the discoveries reported in the study cannot be reproduced by others. The phyloseq package (<https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html>) is highly useful for data preparation, though the authors may consider using an alternative comparative software package if preferred. The authors can refer to the following website if they wish: <https://github.com/angelolimeta/Gut-microbiome-immunotherapy>

Response: We thank the reviewer for emphasizing the importance of data transparency. We have deposited the processed microbiome data (Feature Table, Taxonomic Table, and Phylogenetic Tree) in Zenodo ([10.5281/zenodo.15292903](https://doi.org/10.5281/zenodo.15292903)) and have updated the Data Availability section. Access to privacy-sensitive phenotype data follows GDPR-compliant controlled-access procedures through the Lifelines biobank, as described in the manuscript:

“The raw metagenomic sequencing data from this study are publicly available in the European Genome-Phenome Archive under the Dutch Microbiome Project (EGAS00001005027) and Lifelines-DEEP (EGAD00001001991). The processed microbiome data generated during this study, including the Feature Table (raw counts), Taxonomic Table, and Phylogenetic Tree, have been deposited in Zenodo [doi: [10.5281/zenodo.15292903](https://doi.org/10.5281/zenodo.15292903)]. Due to GDPR and Lifelines biobank regulations, access to privacy-sensitive phenotype data (e.g., clinical and lifestyle information) requires following controlled-access procedures. Researchers can apply for access via <https://www.lifelines.nl/researcher/how-to-apply/apply-here>. Applications are reviewed for compliance with informed consent, GDPR, and Lifelines' non-commercial use policies. Upon approval and completion of a Data and Material Transfer Agreement, data access is provided through the secure Lifelines Workspace or High-Performance Cluster (HPC) environments, with fees set at €3,500 annually for HPC access and €4,500 annually for Workspace access (prorated options available). Full dataset access for replication studies is available free of charge for three months by contacting research@lifelines.nl. Additional information on data access procedures is available at <https://www.lifelines.nl/researcher/how-to-apply>.”

Point 2: Alpha- and beta-diversity analysis: The authors conducted alpha-diversity and beta-diversity analyses using only a few abundance-based indices (Shannon and Bray-Curtis). The authors need to survey other widely used phylogenetic indices such as phylogenetic diversity (PD) for alpha diversity and UniFrac distances for beta diversity.

Response: We sincerely thank the reviewer for highlighting the importance of incorporating phylogenetic diversity metrics. As suggested, we conducted additional analyses using Phylogenetic Diversity (PD) for alpha diversity and weighted UniFrac distances for beta diversity. Both measures showed significant associations with sleep quality (PSQI), social jet lag, and chronotype. We have updated the Results section accordingly, which now reads: “Alpha diversity, as measured by the Shannon index and Phylogenetic Diversity (PD), was significantly associated with PSQI score (Shannon: Beta = -0.015, FDR = 0.024; PD: Beta = -0.256, FDR = 2.31×10^{-4}), social jet lag (Shannon: Beta = -0.017, FDR = 0.024; PD: Beta = -0.185, FDR = 0.020), and chronotype (Shannon: Beta = -0.023, FDR = 0.024; PD: Beta = -0.239, FDR = 0.019) (**Figure S3, Table S3**), which suggests that variations in sleep patterns are reflected in the diversity of the gut microbiome. Beta diversity, assessed using Bray–Curtis and Weighted UniFrac distances, showed significant associations with PSQI (Bray–Curtis: $R^2 = 5.4 \times 10^{-4}$, $P = 0.00001$; Weighted UniFrac: $R^2 = 5.6 \times 10^{-4}$, $P = 0.003$), social jet lag (Bray–Curtis: $R^2 = 4.2 \times 10^{-4}$, $P = 0.0004$; Weighted UniFrac: $R^2 = 5.5 \times 10^{-4}$, $P = 0.007$), and chronotype (Bray–Curtis: $R^2 = 4.3 \times 10^{-4}$, $P = 0.00035$; Weighted UniFrac: $R^2 = 7.1 \times 10^{-4}$, $P = 0.002$) (**Table S4**). These findings indicate that both taxonomic and phylogenetic composition of the gut microbiome are associated with these sleep characteristics.”

We have also updated Supplementary Table S3 and S4, as well as Supplementary Figure 3, to include these new diversity analyses.

Point 3: Statistical Analysis for Repeated Measurements: Since the data come from the prospective Lifelines Dutch Microbiome Project, there may be repeated measurements for the same subjects. If this is the case, the metadata should include both subject IDs and measurement IDs. Treating repeated measurements as independent subjects can artificially inflate the number of discoveries, leading to numerous spurious findings. The authors should employ appropriate statistical methods, such as mixed-effects models, to account for repeated measurements in their analysis.

For PERMANOVA, 1,000 permutations is too few; it is recommended to use 100,000 permutations. Additionally, PERMANOVA does not account for repeated measurements or covariate adjustments; thus, you can consider mixed-effects model-based methods.

Response:

We appreciate the reviewer's thoughtful comment. We would like to clarify that there are no repeated measurements in our dataset; each participant contributed only one microbiome sample and corresponding phenotype record. To make this explicit, we have added a sentence in the Statistical Analysis section, which now reads:

"All analyses were conducted under the assumption of independent observations. Each participant contributed only one microbiome sample and corresponding phenotype record; no repeated measurements were included in the dataset."

Additionally, we have rerun the PERMANOVA analyses with 100,000 permutations, as recommended. The updated results are now reflected in Supplementary Table S4.

Point 4: Mediation analysis: "Employing the mediation package in R" is not at all sufficient. The authors need to disclose the method name and reference. The authors need to disclose how the repeated measurements and covariate adjustments were handled with more specific methodological descriptions.

1. Provide references for the statement: "Variable selection in the regularized mediation model was performed using a lasso-penalized model, with the optimal lambda for the lasso (L1) penalty determined by minimizing the Bayesian Information Criterion through a grid search."
2. Perform mediation analysis for both alpha- and beta-diversity indices as well.

Response:

We thank the reviewer for this helpful comment. In response, we have now revised the Methods section to provide a more detailed and transparent description of our mediation analysis procedures. Regarding repeated measurements, we would like to clarify that this study is based on cross-sectional data, and no repeated measurements were available. All phenotypes and microbiome features were measured at a single time point. In addition to species-level abundance, we now include alpha diversity (Shannon index and Phylogenetic Diversity) and beta diversity (the first 20 principal coordinates from weighted UniFrac distances) as microbial traits in both the univariable and multivariable mediation analyses, as suggested. These revisions are now reflected in the Methods section, and the updated mediation results including diversity metrics can be found in Supplementary Tables 8–14.

"Inferring the putative causal relationship linking sleep, gut microbiome, and diet or disease"

Having established a link between sleep and bacterial composition, we sought to determine whether alterations in sleep patterns influence microbial species abundance and diversity measures, or if the reverse is true. To investigate this, we incorporated dietary patterns, which we hypothesized to be more likely to be an exposure than a consequence to the microbial features, into our mediation network analysis. This analysis tested three pre-defined potential directional relationships: (i) diet → microbiome → sleep, where changes in microbial features potentially cause alterations in sleep characteristics, (ii) diet → sleep → microbiome, and (iii) sleep → diet → microbiome, where changes in microbial features are outcomes of lifestyle modifications.

In the first scenario (**Figure 4a, Table S10**), we observed that most species demonstrated a direct link to either sleep or diet. However, two species, *Clostridia bacterium UC5_1_1E11* and *Clostridia unclassified SGB14844* (both from the unclassified *Clostridia* genus), acted as putative mediators in the diet-to-sleep pathway, suggesting a potential mediation of the effect of coffee consumption on social jet lag. Increased coffee intake was found to boost the abundance of these two species, consequently intensifying the extent of social jet lag. This interesting dynamic between coffee consumption, the two *Clostridia* species, and social jet lag was also observed in the second (**Figure 4b, Table S11**) and third scenarios (**Figure 4c, Table S12**), indicating that fluctuations in social jet lag might impact coffee consumption, or vice versa, thereby altering the abundance of these two *Clostridia* species.

Since these two species appeared in a mediation relationship in all three directions, we could quantify the proportion of mediation based on effect sizes derived from an unpenalized model (**Figure 4d**). By aggregating the effects of UC5_1_1E11 and SGB14844, we discovered that the abundance of these species mediated 10% of the effect of coffee intake on social jet lag. Conversely, social jet lag accounted for only 1.7% of the effect of coffee intake on these species. Notably, coffee intake was found to mediate 59.8% of the effect of social jet lag on these species. These estimates suggest that variation in the abundance of these two *Clostridia* species related to social jet lag may largely reflect differences in coffee consumption.

Along the same line, the connection between many microbial features and sleep characteristics was found to be potentially mediated by specific dietary factors (direction III) (**Figure 4c, Table S12**). Similar to the two *Clostridia* species, many species abundances could also be affected by changes in coffee consumption induced by social jet lag. *Lachnospiraceae GGB3570_SGB4777*, *Ruminococcaceae GGB9623_SGB15076*, and *Clostridium phoceensis* showed an increase following coffee intake, while *Ruminococcaceae GGB9699_SGB15216* exhibited a decrease. Additionally, a preference for a later chronotype was linked with increased alcohol consumption, which in turn led to a reduced abundance of *Coprococcus eutactus*. Poor sleep quality associated with a lower HEI led to an increased abundance of *Flavonifractor plautii*. Nevertheless, findings from direction II still revealed that certain species do exhibit a direct connection with sleep patterns, independent of dietary mediation (**Figure 4b, Table S11**). For example, individuals with a late chronotype displayed elevated levels of *Lachnospiraceae GGB3570_SGB4777* and a reduction in *Clostridium sp. Marseille P3244* and *Clostridium leptum*.

To extend the analysis beyond dietary exposures, we also evaluated whether the gut microbiome could serve as a mediator in associations between sleep characteristics and disease phenotypes, focusing on gastrointestinal symptoms and mental health conditions (Table S13-14). However, we did not identify any microbial features or diversity measures that met the criteria for significant mediation in these models.”

Other Comments:

Point 5: A reference needs to be provided for the following statement: “Before conducting any statistical analyses, we applied inverse rank normal transformation to the sleep indices (PSQI score, ESS score, social jet lag, and MSFsc score) to reduce skewness and improve normality.”

Response: We appreciate the reviewer's attention to detail. We have now added a reference (Beasley et al., 2009, Behavior Genetics) to support the use of inverse rank normal transformation in the Methods section.

Point 6: The statement "To correct for multiple testing, we estimated the FDR by conducting 1,000 rounds of permutation" is unclear and requires further clarification.

Response: Thank you for pointing this out. To improve clarity, we have moved the description of FDR estimation to the Statistical Analysis section and expanded it to explain the permutation-based approach used. The relevant text now reads: *"A false discovery rate (FDR) was estimated using a permutation-based approach. For each analysis, we shuffled the sleep phenotype labels 1,000 times and recalculated association p-values for each permuted dataset, thereby generating a null distribution of association p-values under the assumption of no true association. To estimate the FDR for a given p-value threshold, we calculated the proportion of p-values under the null distribution that were equal to or smaller than the threshold, divided by the proportion of observed p-values that were equal to or smaller than the same threshold. Associations were considered statistically significant if their empirical FDR was below 0.05, and all tests were two-sided."*

Point 7: The authors can also conduct functional (gene content) analyses in addition to the alpha-diversity, beta-diversity, and species analyses.

Response:

We thank the reviewer for this valuable suggestion. In addition to our taxonomic and diversity-based analyses, we have now conducted species-level profiling to explore functional variation in two key species of interest: *Clostridia* bacterium UC5_1_1E11 and *Clostridia* sp. SGB14844. These species were identified as potential mediators in the relationship between coffee intake and social jet lag. Using HUMAnN 4.0 and MetaCyc pathway annotations, we examined within-species variation in pathway abundance. While this approach provided a more detailed view of microbial function, we did not observe strong or consistent functional signatures related to sleep or dietary traits. These results highlight the current challenges in translating species-level associations into mechanistic insights. The results of the functional profiling are now included in the revised manuscript and presented in Supplementary Table 15. We have also added a discussion of the limited functional signals observed, as noted in the Discussion section

Method: ***"Functional and phylogenetic profiling of Clostridia species"***

To further explore Clostridia bacterium UC5_1_1E11 and Clostridia sp. SGB14844, we performed species-resolved pathway and phylogenetic analyses. For functional profiling, we used HUMAnN 4.0 to quantify MetaCyc pathway abundances within each species. A total of 106 pathways were detected across the two species (48 in SGB14844 and 58 in SGB14895). Associations between CLR-transformed pathway abundances and phenotypes (coffee intake and social jet lag) were tested using linear models adjusted for age, sex, BMI, DNA concentration, sequencing batch, post-QC read count, Bristol Stool Scale, and defecation frequency. Analyses were performed separately per species among individuals with complete data (n = 630 for SGB14844; n = 77 for SGB14895).

To evaluate whether phylogenetic relatedness among strains could help explain phenotype associations, we reconstructed strain-level phylogenies using StrainPhlAn with minimum marker detection thresholds set to 10 across samples. We then fit phylogenetic generalized linear mixed models using the anpan R package⁴², modeling phenotypes as a function of covariates and a phylogenetic random effect under a Brownian motion assumption. Model fit was assessed by comparing the expected log predictive density

(ELPD) of each phylogenetic model to a null model without the phylogenetic term, using Pareto-smoothed leave-one-out cross-validation. A phylogenetic signal was considered statistically supported if the ELPD difference (ΔELPD) between the null and phylogenetic model was less than -4 and the 95% confidence interval ($\Delta\text{ELPD} \pm 2$ standard errors) did not include zero.”

Result: “To further investigate the functional and evolutionary basis of these two *Clostridia* species, we conducted exploratory analyses of their metabolic pathways and phylogenetic relationships. Despite their putative role in mediation, we identified no significant ($\text{FDR} < 0.05$) metabolic pathways or phylogenetic conservation of these species’ associations with social jet lag or coffee intake (**Table S13–14**). While their functional roles remain unclear, these species could influence circadian rhythms through unmeasured metabolites or indirect mechanisms, warranting deeper functional profiling in targeted studies.”

Discussion: “In particular, two *Clostridia* species, UC5_1_1E11 and SGB14844, were consistently involved in the interplay between coffee consumption and social jet lag across multiple predefined mediation scenarios. Together, the abundance of these two species mediated approximately 10% of the association between coffee intake and social jet lag. Although the majority of the effect is likely direct, or mediated through other factors, the observed mediation suggests that these species may play a meaningful biological role and may represent potential targets for mitigating the impact of coffee intake on social jet lag. Although these species remain poorly characterized, their membership in class *Clostridia*, which includes many species known to influence host metabolism⁵³ and neuroactive compound production⁵⁴, points to plausible mechanisms involving microbial metabolites affecting sleep regulation. To further explore their potential roles, we examined the pathway-level functional profiles and phylogenetic patterns of these two species. However, we did not identify any specific metabolic pathways or phylogenetic signals that could explain their associations with coffee intake or sleep-related traits. These null findings may reflect the relatively low prevalence and abundance of these species in the population, limiting the statistical power to detect subtle biological effects. Nonetheless, their consistent appearance in directional models highlights them as intriguing candidates for future mechanistic investigation.”

Response to Reviewers

The Interplay of Sleep Characteristics with Health factors and Gut Microbiome

Reviewer #1:

The authors thoroughly explained their revisions and efforts, and all comments were addressed satisfactorily.

Reviewer #2:

Thanks to the authors for their efforts in addressing my previous comments. Most concerns have been largely resolved. However, I have two remaining points regarding the authors' responses:

Point 1: This issue relates less to non-normality or outliers, and more to the fundamental nature of the variables themselves. The authors' arguments, such as treating them as continuous and applying transformations, do not sufficiently address the concern that the observed associations may be artifacts without clear biological relevance. I still believe that these methodological choices alone cannot rule out the possibility that the findings lack meaningful interpretation.

Response: We appreciate the reviewer's concern that the observed associations might reflect artifacts arising from the fundamental nature of the sleep variables rather than biology. As with any observational study, we cannot completely rule this out, and we see it as a general limitation of the field. However, we would like to address the validity and biological significance of these variables from several angles.

First, the sleep phenotypes analyzed in our study (Pittsburgh Sleep Quality Index (PSQI), Epworth Sleepiness Scale, social jet lag, and chronotype) are well established and widely used. Using PSQI as an example, this self-report questionnaire was developed in 1989 (1) and has been widely used to assess sleep quality worldwide. It has been translated into 56 languages (2) and is cited in 40,788 studies (3). Furthermore, with the widespread adoption of wearable devices, PSQI scores have been validated against smartwatch-derived measurements. For instance, Ko et al. assessed sleep quality in patients with Parkinson's disease using both PSQI and smartwatch sensors and reported consistent results (4).

Secondly, in our cohort, we tested the sleep measures against non-microbial phenotypes and recapitulated well-described patterns. Social jet lag decreased with age (5). Better diet quality was associated with better PSQI scores (6). Evening chronotype was associated with higher alcohol intake (7). Each of these findings is consistent with prior literature and supports the conclusion that our sleep variables behaved as expected in this dataset. These results are discussed in the Results section (i.e. sleep characteristics

are highly associated with diet, disease, socioeconomics, and other factors) and presented in Table S2. Thus, we believe that these indices do reflect biological facets of sleep health.

Third, to further support robustness, we conducted a targeted replication of previously reported sleep–microbiome associations (with results shown in the table below). Most prior studies used 16S rRNA sequencing and reported associations at the genus or family level. Because our dataset is species-resolved by shotgun metagenomics, we evaluated replication at the same genus/family level to match the resolution of the original reports. For each reported genus- or family-phenotype pair, we applied the same modeling framework as in our primary analyses and tested whether the corresponding taxon in our data showed a same-direction association with the same sleep trait. We observed directional concordance for several taxa (see table below). For example, *Bacteroides* showed positive association ($\beta=0.076$, $p=1.82\times 10^{-4}$) and *Prevotella* negative association ($\beta=-0.134$, $p=0.027$) with PSQI, consistent with earlier reports. *Coprococcus* was also negatively associated with sleep disturbance ($\beta=-0.089$, $p=0.010$). Although the total number of direct replications was modest, this is unsurprising given platform and reference differences (16S vs shotgun), cohort and phenotype variation, and the fact that genus-level tests aggregate species with potentially divergent effects, which can dilute or cancel species-specific signals. Consistent with this, we observed within-genus heterogeneity in our data (see the *Blautia* and *Dorea* examples in the Discussion). Taken together, the concordance we observe at higher taxonomic ranks supports the biological plausibility and validity of the sleep measures and associations reported here.

Study	Study Population	Sample Size	Sleep Trait	Microbiome	Taxa	Estimate	Std.Error	P-value
Grosicki et al. [8]	Young healthy subjects	28	PSQI	16S seq	Blautia (Genus)	0.042	0.030	0.163
					Ruminococcus (Genus)	-0.017	0.035	0.630
					Prevotella (Genus)	-0.134	0.061	0.027
Smith et al. [9]	Healthy adults	26	Actigraphy	16S seq	Lachnospiraceae (Family)	-0.009	0.012	0.457
					Corynebacterium (Genus)	0.004	0.005	0.381
					Blautia (Genus)	0.042	0.030	0.163
Zhang et al. [10]	College students	34	PSQI	16S seq	Firmicutes (Phylum)	0.004	0.007	0.609
					Proteobacteria (Phylum)	0.028	0.014	0.051
					Elusimicrobia (Phylum)	-0.018	0.016	0.274
					Tenericutes (Phylum)	-0.115	0.045	0.011
					Elusimicrobia (Class)	-0.035	0.016	0.033
					Mollicutes (Class)	-0.135	0.048	0.005
					Erysipelotrichia (Class)	-0.006	0.034	0.861
Wang et al. [11]	Preschool aged children	143	Actigraphy	16S seq	Bifidobacterium (Genus)	-0.012	0.037	0.742
					Bacteroides (Genus)	0.076	0.020	1.82E-04
					Lachnospiraceae (Family)	-0.009	0.012	0.457
					Blautia (Genus)	0.042	0.030	0.163
					Coprococcus (Genus)	-0.089	0.035	0.010
Zhang et al. [12]	Patients with MDD and healthy controls	81 (36 MDD, 45 HC)	PSQI	16S seq	Dorea (Genus)	-0.044	0.036	0.222
					Intestinibacter (Genus)	0.011	0.020	0.582
					Coprococcus (Genus)	-0.089	0.035	0.010
Mairinger et al. [13]	Patients with psychiatric disorders and healthy controls	103	PSQI	16S seq	Ellagibacter isourolithinifaciens (Species)	Not detected		
					Senegalimassilia faecalis (Species)	0.007	0.020	0.739
					uncultured Blautia sp. (Species)	Not specified		
					Senegalimassilia (Genus)	0.023	0.039	0.544
					uncultured Muribaculaceae (Genus)	Not specified		

Tanaka et al. [14]	Patients with depression and anxiety disorders	40	PSQI	16S seq	Bacteroides (Genus)	0.076	0.020	1.82E-04
Carasso et al. [15]	Medication-free individuals	91	Chronotype	Metagenome	Alistipes (Genus)	0.034	0.026	0.186
					Lachnospira (Genus)	-0.034	0.026	0.186
					Alistipes putredinis (Species)	0.048	0.041	0.242
					Lachnospira pectinoschiza (Species)	-0.048	0.041	0.242

Taking all of our lines of evidence above together, we believe that we have addressed the reviewer's concern about the validity of our sleep parameters and the potential biological relevance of our signals. Nevertheless, we acknowledge our study does not provide definitive evidence about causality or underlying biological mechanisms and that self-reported questionnaire data have inherent limitations. We have now strengthened our discussion of these limitations.

Discussion section (L636-642): *"As mentioned, one of the major limitations of our study is its cross-sectional design, which prevents us from establishing the temporal order of events. This is particularly relevant for mediation analysis, where a clear sequence of exposure, mediator, and outcome is required to support causal interpretation. In addition, although we adjusted for a broad set of known covariates, we cannot exclude the possibility of unmeasured confounding. Our mediation results should thus be viewed as exploratory and interpreted with caution. Moreover, the reliance on self-reported data for both sleep patterns and dietary habits introduces potential reporting bias. Although self-reporting is valuable for gathering data in large-scale studies, it is prone to inaccuracies and personal biases. Objective measurements, such as actigraphy for sleep and detailed diet logs or biomarkers for dietary intake, would enhance the validity of results."*

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Point 2: The authors appear to have rotated the directionality of exposure, mediator, and outcome in their mediation analysis, which is not consistent with the assumptions of classical mediation frameworks. This reorientation also compounds concerns about multiple comparison adjustments, particularly in the context of an already high-dimensional analysis. Furthermore, the use of univariate models as a screening step for multivariable modeling is methodologically problematic. This approach inflates the risk of Type I errors (false positives) due to multiple comparisons and reduces power to detect true associations in a joint model (Type II errors). As a result, the final models may be

biased—either overestimating the importance of certain predictors or overlooking key relationships.

Response: Thank you for the thoughtful comment. We appreciate the opportunity to clarify. We hypothesized that there is a bidirectional effect between the gut microbiome and sleep and tested this hypothesis using mediation analysis. This hypothesis is evidence-based (16,17). We did not rotate the roles of exposure, mediator, and outcome post hoc or based on statistical significance. Roles were fixed a priori, defined by a pre-specified directed acyclic graph consistent with the hypothesized direction of effect, and each direction was evaluated as a separate pre-specified model. In human cohort studies, it is common to define a set of competing directional models, including bidirectional paths, and to evaluate them transparently. In particular, it is common practice to evaluate both directions in scenarios where biological feedback is plausible (18,19), and some studies have even evaluated direction-sensitive serial orders (20,21). Our approach follows this practice.

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We agree that prefiltering variables using univariate p-values might bias subsequent multivariable selection. In this revision, we have now removed the univariate screen and instead embedded the LASSO-based penalized multivariate mediation in a feature subsampling stability selection framework. This replaces hypothesis test p-values with stability metrics that prioritize replicability across many subsampled fits, reducing the chance that a single noisy or collinear configuration drives selection. For each direction, 10,000 iterations were executed. Overall, our pipeline now relies on joint penalization with stability selection: the penalty shrinks spurious effects and subsampling mitigates switching among highly correlated features. We believe this yields more robust and reproducible results.

We have now described this in the Methods Section (L264-290):

*“Multivariable mediation was evaluated across five pre-specified causal directions spanning diet- and disease-related hypotheses using the regmed package in R for regularized mediation analysis via penalized structural equation modeling⁴⁰. For abundance-based analyses, to reduce bias from feature preselection and enhance robustness, lasso-penalized mediation models were embedded within a subsampling-based stability selection procedure. For each direction, 10,000 iterations were executed. In each iteration, 30 candidate microbial species were randomly sampled from the full candidate set and a lasso-penalized mediation model was fit over a grid of L1 penalty strengths ($\lambda \in \{0.02, 0.015, 0.01, 0.005\}$)⁴¹. The penalty minimizing the Bayesian Information Criterion (BIC) was selected. Linear mediation was parameterized as $M = \alpha X + \dots$ and $Y = \beta M + \delta X + \dots$, where X denotes the exposure, M the putative mediator, and Y the outcome. A species was deemed selected in an iteration if, for the species-as-mediator direction, both $\alpha \neq 0$ and $\beta \neq 0$, or if, for the species-as-outcome direction, both $\beta \neq 0$ and $\delta \neq 0$. For each species, the number of eligible runs (iterations in which the species was included), selected runs (iterations meeting the selection rule), and the selection rate (selected/eligible $\times$ 100%) were recorded (**Table S8**). Within each direction, species with a selection rate $>10\%$ were retained, and a final model with these species was fit. For composition-based analyses, we did not perform stability selection; instead, we fit one lasso-penalized model per direction to the full set of diversity metrics, using the same λ grid and BIC-based tuning described above. To reduce shrinkage bias, we refit the multivariable mediation model on the reduced set (variables with nonzero coefficients at the BIC-selected λ) and then obtained effect-size estimates from an unpenalized model restricted to those variables. For each pathway, the proportion of mediation attributable to the mediator was quantified based on the effect sizes from the non-penalized model. This proportion was determined by dividing the mediated effect (the product of the pathways from X to the mediator and from the mediator to Y) by the total effect (the sum of the mediated effect and any direct effect).”*

By applying the stability selection, two species (Clostridia unclassified SGB14844 and Clostridia bacterium UC5_1_1E11) were repeatedly selected ($\geq 50\%$ of 10,000 iterations) in each of the three mediation directions. These features were retained in the refitted model and are highlighted in **Fig. 4** with the estimated proportions mediated. In addition, we report a broader set of potential candidates (selection frequency $\geq 10\%$) as network visualizations in **Supplementary Fig. S7** to aid hypothesis generation and future validation. Together, these results indicate that the strongest microbial features are stable under resampling, while the extended set provides testable targets for follow-up. That said, stability alone does not imply causality; some features may reflect correlated or underlying processes rather than direct effects. We therefore present them as replicable signals to guide future studies, rather than as definitive causal claims.

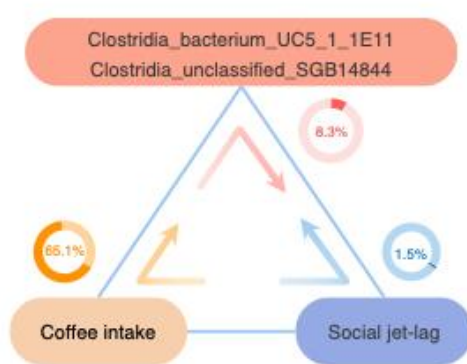


Figure 4. Mediation among coffee intake, social jet lag, and Clostridia taxa.

The inferred causal relationship between Clostridia bacterium UC5_1_1E11, Clostridia unclassified SGB14844, coffee intake, and social jet lag. The percentage shown quantifies the proportion of mediation in this relationship.

Reviewer #3

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

There are several critical issues with the provided datasets, including the feature table, taxonomic table, and phylogenetic tree. Most notably, the feature table does not contain raw count data, contrary to the authors' claim, and the feature IDs are inconsistent across the three datasets. The manuscript states that there are 6,941 subjects, yet the feature table includes 8,355, with no explanation or method provided to extract the 6,941 subjects referenced. Furthermore, without metadata, it is impossible to verify the authors' claims. As a result, the findings presented in the manuscript amount to unsubstantiated

assertions. The absence of transparent data reporting is detrimental to scientific integrity. Without proper data verification, I cannot recommend this manuscript for publication.

Response: We appreciate the reviewer's close attention to data transparency.

Regarding identifiers, **FeatureID** is consistent across the taxonomy table and feature table. In the phylogenetic tree, we use the shorter **SGB_ID** as tip labels for readability. A one-to-one mapping between **FeatureID** and **SGB_ID** was provided in **SGB_tree_annotation.csv**.

We apologize for the confusion around “raw counts.” In the feature table, we provide MetaPhlAn-derived relative abundances, not raw counts. MetaPhlAn estimates taxon abundance from clade-specific markers, yielding compositional data that are directly comparable across heterogeneous sequencing depths and appropriate for our centered log-ratio (CLR)–based and diversity analyses. To avoid confusion, we have revised the text to state “MetaPhlAn relative abundances” rather than “raw counts”.

The feature table includes all post-QC samples (n = 8,355), whereas the analysis cohort (n = 6,941) is the subset with complete sleep variables and required covariates. To ensure full reproducibility, we now provide an explicit ID list (**analysis_cohort_ids.txt**) so readers can filter the matrices to exactly 6,941 samples.

Finally, the individual-level metadata are owned by the Lifelines biobank. Like any other researcher, we access these data under GDPR and participant-consent restrictions and therefore cannot redistribute them. Within these constraints, we maximize transparency and reproducibility by sharing all allowable feature matrices and derived outputs, and by providing step-by-step instructions that enable qualified researchers to obtain the corresponding data directly from Lifelines. For replication of published findings, the biobank offers a 3 month, no-cost access window, and we are happy to point applicants to the exact variables and project fields needed to reproduce our results.

The Data Availability section now reads as follows (L697-714):

“The raw metagenomic sequencing data from this study are publicly available in the European Genome-Phenome Archive under the Dutch Microbiome Project (EGAS00001005027) and Lifelines-DEEP (EGAD00001001991). The processed microbiome data generated during this study, including the Feature Table (MetaPhlAn-derived relative abundances), Taxonomic Table, and Phylogenetic Tree, have been deposited in Zenodo [doi: [10.5281/zenodo.15292903](https://doi.org/10.5281/zenodo.15292903)]. Due to GDPR and Lifelines biobank regulations, access to privacy-sensitive phenotype data (e.g., clinical and lifestyle information) requires following controlled-access procedures. Researchers can apply for access via <https://www.lifelines.nl/researcher/how-to-apply/apply-here>. Applications are reviewed for compliance with informed consent, GDPR, and Lifelines' non-commercial use policies. Upon approval and completion of a Data and Material Transfer Agreement, data access is provided through the secure Lifelines Workspace or High-Performance

Cluster (HPC) environments, with fees set at €3,500 annually for HPC access and €4,500 annually for Workspace access (prorated options available). Full dataset access for replication studies is available free of charge for three months by contacting research@lifelines.nl. Additional information on data access procedures is available at <https://www.lifelines.nl/researcher/how-to-apply>. ”