nature portfolio

Peer Review File

Gut microbiome features associated with Bifidobacterium colonization predict personalized probiotic persistence patterns

Corresponding Author: Dr Tarini Ghosh

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)
I read with interest the manuscript: “Identifying the non-Bifidobacterial hallmarks of a Bifidobacterium-receptive gut microbiome” by Goswami et al. The study presents a large-scale analysis of over 50,000 gut microbiome datasets from 46 countries to elucidate ecological and functional factors associated with Bifidobacterium colonization. Using machine learning and functional genomics, the authors propose an ecological-based scoring system. Association-Scores and Receptive-Scores, to describe the association and receptivity of gut microbiomes to Bifidobacterium species. The study provides valuable insights into the global ecology of Bifidobacterium, integrating both cross-sectional and interventional data. The scale and analytical depth of this work are commendable, as is the introduction of an innovative framework to quantify microbial receptivity to Bifidobacterium strains
However, several aspects are not integrated that limit the intepretability of the study

Major Comments

1) Many of the public datasets used lack critical metadata that have been associated with Bifidobacterium such as dietary intake, medication use (notably antibiotics), and host lifestyle or clinical background. These missing variables could confound observed associations. The authors should try to identify studies to account for such limitations.

2) The association between Bifidobacterium abundance and overall microbiome alpha-diversity should be examined, and possibly added as a covariate in models

3) The studies relies on Bifidobacterium and major association to non-Bifidobacterium taxa. How that compared to other taxa (example Bacteroides versus non-Bacteroides …)

4) Incorporating a quantitative microbiome profiling (QMP) data is key and may improve robustness. Such public datasets (Metcardis) can be used to assess how quantitative data could possibly support findings

5) Given the importance of Bifidobacterium in health, it would be valuable to explore how the Receptive-Score relates to clinical outcomes where available

(Remarks on code availability)
I am not able to analyse codes

Reviewer #2

(Remarks to the Author)
The manuscript describes the exploration of the link between a certain non-bif composition of the microbiome and the occurrence and persistence of bif taxa in the human gut microbiome. The topic is timely and very interesting so as part of the results.

The overall study suffers from a too strong methodological shape and descriptions and from lack of clear conclusions. The discussion is to be completely rewritten as it does not currently give interpretation of the results or a clear message to the reader.
I am generally very positive towards this work but I think that a great effort to improve the manuscript is needed to make it a good and useful piece for the gut microbiome community.

Abstract a bit too methodological e vague in terms of results and potential impact. It should be more focused on the links found, on the Non Bif and Bif (specific, in terms of species involved) links etc.

272-314. This section is not clear and the actual value of the results is doubtful. The whole section (including figure 4) should be revised to give a straight and simple message to the reader. At present, it seems that the association with disease was investigated but the authors are not sure of what the results can mean. Is there a clear association between a pair non-Bif/Bif taxa and disease? If yes, please revise dramatically to make it clear. If not, please consider removing the entire section.

323-325. The oligosacch trial is too different in principle, it should be left out.
IN addition, for this part of the study the authors must opt for keeping only WGS datasets leaving out 16S. Too much variability is not advisable here.

426-462. This part is far too preliminary. There's no effort in exploitation of the findings, which remain a very basic information with no possible clue on the real involvement of the functions in predisposing the subjects in Bif receptivity.

483-495. These results are important. However, they are not discussed but only suumarized here. The authors need to make a further effort to give the reader their interpretation of the results and their global impact/meaning. Among others, what is the meaning of the association to butyrate producers?

497-508. This is not a discussion, but rather an additional summary of a part of the study.

509-515. Lack of discussion, see above. What are the possible mechanistic links mentioned?

Minor points
Be consistent: taxa, traits, representations, features all wording to express mainly the same things, basically microbiome composition.

99-100. What do the authors mean here by "representation" of Bifidobacterium members ?
103. I am not sure a question mark is needed here.
115-120. Receptive score must be explained here in its biological and impact meaning to be clearly interpreted by the reader.
204-206. Unclear phrase.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have read the rebuttal of the manuscript entitled "Identifying the non-Bifidobacterial hallmarks of a Bifidobacterium-receptive gut microbiome" by Goswami et al. The authors have performed a great job in complementing the analysis by several key parameters such as confounders and quantitative approach for instance.
The paper looks very heavy in figures (they are very dense). I would actually propose that Figure S1 is integrated as a main figure (Fig 1) and move one of the dense figure to supp info
The discussion can add a more specific section of the novelty in term of ecology beyond the receptive score

(Remarks on code availability)
I am not skilled to check codes

Reviewer #2

(Remarks to the Author)
The authors have done a satisfactory job in addressing all the criticisms highlighted in the previous report.

(Remarks on code availability)

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Response to reviewers

Reviewer #1

Comment 1. I read with interest the manuscript: "Identifying the non-Bifidobacterial hallmarks of a Bifidobacterium-receptive gut microbiome" by Goswami et al. The study presents a large-scale analysis of over 50,000 gut microbiome datasets from 46 countries to elucidate ecological and functional factors associated with Bifidobacterium colonization. Using machine learning and functional genomics, the authors propose an ecological-based scoring system. Association-Scores and Receptive-Scores, to describe the association and receptivity of gut microbiomes to Bifidobacterium species. The study provides valuable insights into the global ecology of Bifidobacterium, integrating both cross-sectional and interventional data. The scale and analytical depth of this work are commendable, as is the introduction of an innovative framework to quantify microbial receptivity to Bifidobacterium strains. However, several aspects are not integrated that limit the intepretability of the study

Response: We thank the reviewer for these positive comments. We have performed additional investigations and incorporated further analyses to enhance the interpretability of our findings and investigate the effect of other covariates like diet, medications, clinical biomarkers, and other microbiome covariates like alpha-diversity, quantitative microbiome profiles (including microbial load) on the association-patterns of non-Bifidobacterial lineages on the major Bifidobacteria.

Comment 2. Major Comments

Many of the public datasets used lack critical metadata that have been associated with Bifidobacterium such as dietary intake, medication use (notably antibiotics), and host lifestyle or clinical background. These missing variables could confound observed associations. The authors should try to identify studies to account for such limitations.

Response: We thank the reviewer for this excellent suggestion. We had already incorporated the effect of host life-style represented by industrialization status groups as this was the only information consistently available across all cohorts. To a degree, this status is a useful overall proxy for some relevant confounders.

Furthermore, we have now ***performed additional validations indicating that the association patterns of the non-Bifidobacterial members with a majority of Bifidobacterial features remain stable even after adjusting for diet, medications, host clinical characteristics and diseases.*** The summary of our findings is provided below.

Some datasets contained subject information pertaining to dietary intake, medications and clinical background (i.e. clinical marker profiles of the host) (as suggested by the reviewer) (listed in the new **Table S15**). The challenge was both the metadata schemas and measured variables differed substantially between cohorts. To address this heterogeneity, we grouped metadata variables into three categories: diet, medication, and clinical markers. We summarized variations for a given metadata using rPCA and PCoA, retaining the top three components from each method (which together captured on average 73.47% of total variation across all cohort-metadata combinations).

We then recalculated associations between non-Bifidobacterial taxa and Bifidobacteria features as partial correlations, adjusting for these six metadata components. Partial correlations are measurement of strength of associations between two properties after adjusting/controlling the effect of one (or more) confounding variables. This yielded metadata-adjusted Association Scores that account for major confounding variation (listed in **Table S16**). ***Correlating these metadata-adjusted-Association-Scores for the different metadata with the overall Association-Scores (for the specific cohort-types) yielded significant positive***

***correlations in 47 out of the 48 scenarios (Figure 5B), indicating significant similarities in the adjusted and unadjusted association patterns*.**

The similarities were especially strong for five Bifidobacterial features (*B. longum/adolescentis/breve/dentium* relative abundances and the Bifidobacterial detection rates) (average correlation = 0.56). The other four Bifidobacterial taxa showed significant correlations across a majority of scenarios albeit with weaker association-strengths. ***These observations indicate that these metadata have a limited influence on the majority of associations between non-Bifidobacterial taxa and Bifidobacteria.***

This finding is provided in **Text S3** and **Figure S18C**, **Figure 5B**.

Pertaining to disease-groups, we had previously included the investigation of the different disease-groups in the adult/senior sub-groups of the IndustrializedUrban cohorts. We further expand on this investigation in the revised version (results shown in **Figure 4**; **Figure S16** and non-Bifidobacteria v/s Bifidobacteria Association-Scores in Controls and different disease-groups listed in **Table S13**). For most Bifidobacterial features (except *B. catenulatum*/*animalis* relative-abundance) and most diseases, Association-Scores of non-Bifidobacterial taxa with Bifidobacterial features in non-diseased controls showed significant positive correlations with those observed in disease cohorts, indicating largely conserved association-patterns (**Figure S16**). As for all other metadata, the strongest reproducibility was observed for the relative-abundances of *B. adolescentis*, *B. longum*, *B. dentium*, and *B. breve*, as well as for overall *Bifidobacterial* detection. This was followed by *B. pseudocatenulatum* and *B. bifidum*, which showed significant positive correlations between control-derived Association-Scores and those from four disease-groups. ***This indicates that, for most of the diseases and the majority of Bifidobacteria, non-Bifidobacterial taxa showing higher Association-Scores with a given Bifidobacterial feature in controls also showed higher Association-Scores with the same feature across majority of disease-groups***. This message appears clearly in the v2 submission.

Finally, we observed ***several consistent patterns between Bifidobacterial taxa and non-Bifidobacteria that were observed reproducibly irrespective of the approach utilized to compute the Association-Scores (overall; controls and diseased-groups; diet-/medication-/clinical-metadata-adjusted; diversity/evenness/estimated microbial load-adjusted,*** *please refer to our responses to comments 3 and 6 for the microbiome-property-adjusted scores****)*** (**Figures 3, 4, 5, S18, S19** and **S21**; **Tables S11-S16**). We highlight and summarize these consistent associations in **Figure 5C.**

Comment 3. The association between Bifidobacterium abundance and overall microbiome alpha-diversity should be examined, and possibly added as a covariate in models

Response: We thank the reviewer for this suggestion, and to incorporate this in the revised manuscript we have performed additional analyses to address three related aspects, which are described below along with their corresponding results:

a. ***Association of microbiome alpha-diversity (both Shannon Index and Pielou's-Evenness Index) with the nine Bifidobacterial features***: We computed Shannon Index and Pielou's-Evenness Index (both for each microbiome). We also estimated the Microbial Load (only for WGS datasets) (please refer to our response on the quantitative microbiome profiling below). We then investigated the association strengths of these microbiome-level properties with the nine Bifidobacterial features. First, in the Random Forest models, while ***Shannon/Pielou's-Evenness indices showed high predictive power in general for the different Bifidobacterial features, the predictive power of estimated Microbial Load was relatively low*** **(now shown in the modified Figure 2; and Figures S4-S11)**.

We then computed the Association-Scores of with the different Bifidobacterial features. ***Shannon/Pielou's-Evenness indices showed consistent positive associations with four***

Bifidobacterial features (overall Bifidobacterium detection and B. longum/adolescentis/pseudocatenulatum relative abundances) in both the Infant and Adult gut microbiomes from IndustrializedUrban cohorts (irrespective of sequencing type), but weaker associations in microbiomes from RuralTribal cohorts (now shown in Figure 3; Tables S5-S12). This was ***also reproduced across controls and disease-groups in the IndustrializedUrban cohorts***.

We have highlighted these new findings in the **Results** section titled 'Non-Bifidobacterial taxa exhibit distinct association-patterns with Bifidobacteria, varying by subject age, lifestyle (industrialization), and microbiome profiling method' and **Text S3**.

b. Effect of microbiome-level Shannon Index, Pielou's-Evenness Index and estimated Microbial Load on the association-patterns between the different Bifidobacterial features and the different non-Bifidobacterial taxa: We tested if the reported associations could be driven by these microbiome properties, namely Shannon Index, Pielou's-Evenness Index, and estimated Microbial Load (for WGS cohorts, please refer to our response to Comment 5 below). To account for these potential confounders, we recomputed Association-Scores using study-specific partial correlations, thereby quantifying relationships between non-Bifidobacterial taxa and Bifidobacterial features after controlling for these microbiome properties (See **Text S3** and **Methods**; **Figure S18A** for methodology). As mentioned in our previous response, partial correlations are measurement of strength of associations between two properties after adjusting/controlling the effect of one (or more) confounding variables. Across cohort types, the resulting microbiome-property–adjusted Association-Scores (**Figure S19)** remained highly consistent with the overall scores (presented in **Figure 3**), showing significant positive correlations in 91 of 95 tested scenarios (**Figure 5A**). Thus, ***taxa exhibiting stronger associations in the unadjusted analysis in general retained stronger associations after adjustment, indicating that these patterns are not driven by overall microbiome diversity or load***. This message appears clearly in the v2 submission.

In addition, we also report the adjusted scores for transparency and for use by interested researchers (**Table S14**).

c. Evaluating the effect of inclusion of diversity measures in the computation of Receptive-Scores on the performance of Robust Linear Regression models to predict post-treatment persistence or increase of the administered Bifidobacteria in the Intervention trials cohorts: Since both Shannon Index and Pielou's-Evenness Index were strongly associated with multiple Bifidobacterium features, we also checked whether including these for the computation of Receptive-Scores could improve the performances of models to predict post-treatment increase/persistence of the corresponding Bifidobacteria in our investigation of the intervention trials cohorts. ***After inclusion of these additional features, the RLR models retained a significant positive association between the Receptive-Score and post-treatment increases in the target Bifidobacterium features in 9 out of 13 combinations, while the LOOCV-based linear regression models showed significant correlations between predicted and observed increased in 10 out of 13 combinations, including the Gomez trial (Table S20), indicating similar performance as the models not including these features***.

Comment 4. The studies relies on Bifidobacterium and major association to non-Bifidobacterium taxa. How that compared to other taxa (example Bacteroides versus non-Bacteroides …)

Response: This is an excellent suggestion. While the focus of the current study was on Bifidobacteria, we have another ongoing large-scale study where we investigate the microbiome receptivity of all major gut lineages using a large-scale diverse microbiome cohort

and utilizing multiple deep-learning based decoder-encoder workflows. We respectfully request to retain the focus of the current manuscript only on Bifidobacteria, while we investigate the latter aspect as part of a separate study.

Comment 5. Incorporating a quantitative microbiome profiling (QMP) data is key and may improve robustness. Such public datasets (Metcardis) can be used to assess how quantitative data could possibly support findings

Response: We sincerely thank the reviewer for this suggestion. We have investigated this in the revised version of the manuscript. As rightly pointed out by the Reviewer, quantitative microbiome profiling (sample-specific microbial load) was available in the MetaCardis cohort[1].

To address the reviewer's query regarding the potential impact of quantitative microbiome profiling (QMP), we ***explicitly evaluated whether accounting for microbial load alters the observed associations***. Following the approach proposed in the original QMP study[1], we obtained ***estimated quantitative microbiome profiles*** (or absolute abundance profiles) by multiplying taxa abundances with the sample-specific microbial load. We then recomputed associations between non-Bifidobacterial taxa and Bifidobacterial features using these QMPs or absolute abundance profiles (**Methods** and **Text S3**).

Importantly, ***the resulting absolute abundance-derived correlations were highly consistent with the corresponding Association-Scores obtained using relative abundances***. Specifically, for WGS-sequenced adult and senior industrialized cohorts, ***significant positive correlations were observed between absolute abundance-derived associations and relative-abundance–based Association-Scores for all nine Bifidobacterial features in adults and for seven features in seniors (Figure S17)***. Moreover, for the five Bifidobacterial features that showed the strongest and most consistent patterns across controls and disease-groups (**Figure 4**; **Figures S16-S17**), correlations were uniformly high (≥0.50), indicating that the observed associations were ***robust and largely independent of whether relative or absolute abundances are used***. Thus, this message appears clearly in the v2 submission.

In the study on the MetaCardis cohort, the authors had also developed and provided a machine-learning model to predict overall microbial load from the microbiome-level taxonomic abundance profile[1]. Utilizing the same methodology, we ***then predicted the estimated microbial loads across all WGS sequenced gut microbiomes*** (given that the original model was developed for the WGS gut microbiomes). Subsequently, we investigated the effects of the different microbiome-level properties (Shannon Index, Pielou's-Evenness Index and estimated Microbial Load, the later computed only for the WGS cohorts) using the partial correlation-based approach (as summarized in our response to comment 3) (methodology described in **Figure S18A** and described in detail in **Text S3**). As mentioned in our previous response, in 91 of the 95 tested scenarios, the ***correlations were significantly positive***, indicating that non-Bifidobacterial taxa showing unadjusted higher Association-Scores (**Figure 3**) with a given Bifidobacterial feature also showed higher microbiome-property-adjusted Association-Scores (**Figure S19**) with the same feature, thus indicating that ***overall microbiome properties exert limited influence on these association-patterns*** (**Figure 5A; Figure S18B**).

Comment 6. Given the importance of Bifidobacterium in health, it would be valuable to explore how the Receptive-Score relates to clinical outcomes where available

Response: We thank the reviewer for this suggestion. However, clinical metadata were only available for two of the trials (RamakrishnanM_2025 and BaZ_2021) investigated here in[2,3]. The sample numbers were also lower. Despite this limitation, we investigated the effect of Receptive-Scores in these trials with changes in different clinical attributes (**Figure S24**).

For eight of the 10 subjects constituting the RamakrishnanM_2025 trials, there was a negative correlation between Receptive-Scores (for *B. adolescentis*) and increase in gastrointestinal symptoms (Diarrhoea incidence: R = -0.76; P-value = 0.02 [Significant]; Abdominal Pain: R = -0.58; P-value = 0.13; Faecal Urgency: R = -0.41; P-value=0.3) (**Figure S24 A-E**). Although we could not attain statistical significance because of the limited cohort-size, there was a significant positive correlation between Receptive-Score and an overall reduction in all gastrointestinal symptoms (R=0.73; P-value=0.038). Similarly, for the BaZ_2021 B. animalis trial, higher Receptive-Scores at pre-treatment were associated with reduction in multiple markers of inflammation like CD3-CD8 ratio, Interleukin-2 (IL2), Tumor-Necrosis-Factor (TNF) and Interleukin-6 (IL6). Thus, for the limited set of subjects where the clinical response data were available, ***higher baseline Receptive-Scores were associated with better clinical responses*** (**Figure S24 F-I**). However**, *substantially larger numbers of intervention trials with detailed clinical measurements will be required to rigorously evaluate this potential.*** We have now acknowledged this caveat in the Discussion section.

Comment 7. Reviewer #1 (Remarks on code availability):
I am not able to analyse codes
Response: We have uploaded all the codes and relevant data after this revision to GitHub repository (https://github.com/Sourav-MiRe/Project_Bifidobacterium) and Code Ocean (https://codeocean.com/capsule/7280337/tree) and it has also been verified by the Code Ocean team.

Reviewer #2
Comment 1. The manuscript describes the exploration of the link between a certain non-bif composition of the microbiome and the occurrence and persistence of bif taxa in the human gut microbiome. The topic is timely and very interesting so as part of the results. The overall study suffers from a too strong methodological shape and descriptions and from lack of clear conclusions. The discussion is to be completely rewritten as it does not currently give interpretation of the results or a clear message to the reader. I am generally very positive towards this work but I think that a great effort to improve the manuscript is needed to make it a good and useful piece for the gut microbiome community.
Response: We apologize for the strong bioinformatic bias (but which also reflects bioinformatic strength) and lack of clearer biological conclusions in v1 manuscript. We also apologize for the lack of biological perspectives in the previous version. We have extensively edited some of the results, sought to identify strong and clearly consistent association-patterns between non-Bifidobacterial taxa and different Bifidobacterial features that are consistently observed across host statuses. We have tried to link our investigations of the functional features to metadata associations (for which evidence was available and compelling). We have also completely reframed the Discussion section to discuss these links. We hope the reviewer will find our revisions satisfactory.

Comment 2. Abstract a bit too methodological e vague in terms of results and potential impact. It should be more focused on the links found, on the Non Bif and Bif (specific, in terms of species involved) links etc.
Response: We apologize for this. We have now completely modified the Abstract section to also highlight the consistent links between specific Non-Bif and Bifs. We have also graded the Bifidobacteria based on their links with the health-associated non-Bifidobacterial gut markers. We have added this to the revised abstract which now reads as follows:

"Bifidobacteria are key health-associated members of the human gut microbiome and are widely used as probiotics, but their colonization success varies substantially between individuals, partly due to baseline microbiome composition.

We analyzed 51,244 gut microbiomes from 149 cohorts (45 countries) to identify non-Bifidobacterial taxa associated with the Bifidobacterial features. We observed several consistent and age-/life-style-specific association patterns of different non-Bifidobacterial taxa with the different Bifidobacteria. Multiple Bifidobacteria showed positive associations with butyrate-producing *Firmicutes* and *Collinsella*; negative associations involved pathobiont-*Firmicutes* and specific *Bacteroidota* taxa. *B. adolescentis* and *B. breve* showed the strongest positive and negative associations, respectively, with health-associated adult gut microbiome members.

We quantified these relationships as Association-Scores, stratified by age/life-style/sequencing-strategy/disease, which were significantly reproducible after adjusting for multiple microbiome-linked, host life-style/clinical covariates, and predictable using species-specific genomic functions. We used these Association-Scores to derive microbiome-level Receptive-Scores that quantify how permissive a baseline microbiome is to increase or persistence of a given *Bifidobacterium*. In an external dataset of eight *Bifidobacterium* interventions (N=1,633 gut microbiomes), Receptive-Scores combined with baseline abundance of the administered Bifidobacteria significantly predicted post-treatment persistence/increase in 69.23% of trial-probiotic pairs. Together, this work identifies microbiome features governing Bifidobacterial colonization and provides tools to predict personalized probiotic responses."

Comment 3. 272-314. This section is not clear and the actual value of the results is doubtful. The whole section (including figure 4) should be revised to give a straight and simple message to the reader. At present, it seems that the association with disease was investigated but the authors are not sure of what the results can mean. Is there a clear association between a pair non-Bif/Bif taxa and disease? If yes, please revise dramatically to make it clear. If not, please consider removing the entire section.

Response: We apologize for the lack of clarity. The primary objective of including this investigation was to answer two specific questions:

A) ***To what extent do the associations between non-Bifidobacterial taxa and the different Bifidobacteria differ between normal controls and different diseases***

B) ***Which interactions between non-Bif/Bif taxa remain stable*** irrespective of host status (controls versus the different diseased-groups)? These will be the set of genuine microbe-to-microbe association-patterns that are putatively not affected by the status of the host.

To clarify the value, we have now revised this section dramatically (including the display items) to specifically answer these questions. The section has been restructured and ***renamed as 'Control and disease-groups show similar association-patterns between non-Bifidobacterial taxa and Bifidobacteria***'.

We have focused on six major diseases (containing gut microbiome profiles in >= 7 study-cohorts). For each of these diseases, we computed the Association-Scores of the non-Bifidobacterial taxa with the different Bifidobacterial features as described in the previous section (as in **Figure 3A**; Methods; Association-Scores for each disease-group provided in **Table S13)**, and compared the same with those obtained with respect to the non-diseased controls from the same study-cohorts (**Figure S16**). For most Bifidobacterial features (except *B. catenulatum*/*animalis* relative-abundances) and most diseases, ***Association-Scores of non-Bifidobacterial taxa with Bifidobacterial features in non-diseased controls showed***

significant positive correlations with those observed in disease cohorts, indicating largely conserved association-patterns (Figure S16).

Next, we ***focused on identifying associations that remain conserved across host status***. The strongest reproducibility was observed for the relative abundances of *B. adolescentis*, *B. longum*, *B. dentium*, and *B. breve*, as well as for overall *Bifidobacterium* detection. This was followed by *B. pseudocatenulatum* and *B. bifidum*, which showed significant positive correlations between control-derived Association-Scores and those from four disease-groups. This indicates that, for most of the diseases and the majority of Bifidobacteria, non-Bifidobacterial taxa showing higher Association-Scores with a given Bifidobacterial features in controls also showed higher Association-Scores with the same feature across majority of disease-groups. ***The revised manuscript now states this clearly***. **Figure 4** and **Figure S16**.

We also observed several 'group-agnostic' consistent patterns in this investigation that mimicked those we observed for the overall Association-Scores (**Figures 3-4**). *B. adolescentis*, followed by *B. longum* and the overall *Bifidobacterium* detection showed consistent positive associations with 'health-associated' butyrate-producing Firmicutes. Similar patterns were observed to a lesser extent for *B. pseudocatenulatum* and *B. bifidum*. *B. adolescentis* also showed multiple negative associations with the pathobiont Firmicutes (**Figure 4**; also shown in **Figure 3**), suggesting the strongest association of this taxa with health-associated microbiome members. The particularly strong associations seen in disease-specific contexts further underscore their potential role as beneficial probiotic organisms (especially *B. adolescentis/longum*) capable of contributing to the amelioration of diverse pathological conditions (**Figure 4**).

At the other end of the spectrum were *B. breve* and the pathobiont *B. dentium*, that showed the opposite pattern with clear links to a putatively detrimental microbiome (irrespective of control/disease-groups): consistent negative associations with 'health-associated' Firmicutes and overall microbiome diversity and evenness and to a lesser extent with normal-gut-associated *Bacteroidota* members; multiple positive associations with disease-associated taxa (*B. breve* with pathobiont Firmicutes; *B. dentium* with oral-taxa that have been linked to multiple diseases[4]). Two other consistent patterns were positive associations with other *Actinobacteria* members (observed especially for detection and *B. longum*, *B. adolescentis*, and *B. pseudocatenulatum*) and Shannon/Pielou's-Evenness indices (observed especially for *B. longum*, *B. adolescentis*) (**Figure 4**). Thus, the different Bifidobacteria could be graded based on their associations with the non-Bifidobacterial markers of a health-associated microbiome, with *B. adolescentis* showing the strongest link with a health-associated state and *B. breve* and *B. dentium* at the other end of the spectrum. These findings have been clearly articulated in the revised version of the manuscript.

Comment 4. 323-325. The oligosacch trial is too different in principle, it should be left out. IN addition, for this part of the study the authors must opt for keeping only WGS datasets leaving out 16S. Too much variability is not advisable here.

Response: In accordance with the Reviewer's suggestion, we have removed the GOS-based Looijesteijn et al. (2024) trial that was included in the earlier version of the manuscript. However, we respectfully request that the validation using 16S-based trials be retained. Importantly, Association-Scores are computed separately for 16S and WGS cohorts, such that Receptive-Scores are inherently stratified by sequencing type.

The inclusion of 16S cohorts therefore does not confound the analysis, but instead enables us to assess the robustness and general applicability of the Receptive-Score framework across commonly used microbiome profiling approaches. Retaining these cohorts allows us to demonstrate that the concept of Receptive-Scores captures biologically meaningful patterns

that are reproducible irrespective of sequencing technology, thereby strengthening the generalizability of the proposed framework. We have successfully investigated both 16S and WGS cohorts side by side in some of our previous studies[5,6].

Comment 5. 426-462. This part is far too preliminary. There's no effort in exploitation of the findings, which remain a very basic information with no possible clue on the real involvement of the functions in predisposing the subjects in Bif receptivity.

Response: We acknowledge this comment. Owing to incomplete functional annotation of microbiome-derived genes, it was not feasible to interrogate every identified functional group for specific mechanistic links underlying these interactions. However, ***in our revised analysis, we were able to identify several key functional groups with plausible mechanistic relevance***. These results are summarized below, and included succinctly in the v2 manuscript.

We now identify a set of 162 functional-groups that showed significant association with multiple *Bifidobacterium* features (and were contributed by multiple non-Bifidobacterial taxa) (**Table S21**). We specifically focused on a subset of 68 groups showing positive associations, with either *B. adolescentis*/*B. longum* or *B. breve*/*B. dentium*, that showed the strongest positive and negative associations with health-associated-signatures of non-Bifidobacterial gut microbiome, respectively. Deeper investigations ***identified certain patterns providing insights into the cross-feeding interactions in the gut community*** (now described in detail in **Text S8**; **Figure S26**).

Functional groups showing positive links with *B. adolescentis/longum* abundance were predominantly observed in the genomes of the health-promoting members (majority of them butyrate) of the gut microbiome (now described in detail in **Text S8**; **Figure S26** and summarized in the **Results** section). These included functional-groups like Shikimate dehydrogenase and Pantothenate kinase, which are associated with production of essential aromatic acids (e.g. tryptophan) and the vitamin Pantothenate (and coenzyme-A, a precursor for short chain fatty acids or SCFAs like butyrate). Notably, we identified many functional-groups which are associated with the production of SCFAs or their precursors. These included proteins like alpha-Glucoside sugar transporters (involved in the internalization of simple sugars), GH32 CAZyme (breakdown of fructo-oligosaccharides into fermentable substrates for butyrate synthesis), Threonine biosynthesis functional-groups (precursor for SCFA propionate) and, aspartate aminotransferase (conversion of aspartate to butyrate precursors oxaloacetate and glutamate). The identification of multiple functional groups associated with simple sugar import and SCFA production has important implications for understanding the mechanistic basis of these consistent associations between non-Bifidobacteria and Bifidobacteria (discussed later; please refer to our response to comment 6 below).

We have also highlighted the negative links in the Results section for this investigation. Non-Bifidobacterial functional-groups positively associated with *B. breve/B. dentium*, were enriched for proteins involved in the transport of metal ions (iron, zinc, and manganese) (predominantly observed oral taxa) and glycine betaine, a trimethylamine (TMA) precursor (**Figure S26**). Metal ion uptake/sequestration are strategies employed by certain microbes, particularly pathogens and pathobionts, to outcompete commensals by limiting micronutrient availability, thereby disrupting gut homeostasis, typical of a detrimental microbiome.

This link has also been treated in the Discussions section. In terms of the *B. breve*-associated functional-groups, preclinical studies and human trials have also shown the administration of Bifidobacterial probiotics to be associated with decrease of TMA levels in the gut and serum and the abundances of TMA-producing pathobiont Firmicutes, like *Clostridium asparagiforme/hathewayi/citroniae*[7,8]. This is also observed in our identification of glycine-betaine transporters (negatively linked to *B. adolescentis* abundance), that function

to import precursors of trimethylamine (TMA) which is then converted to trimethylamine-N-oxide (TMAO), consistently linked to diseases such as atherosclerosis, neuroinflammation, and colorectal cancer[9,10]. However, the mechanistic bases of such links still need to be investigated.

Comment 6. 483-495. These results are important. However, they are not discussed but only suumarized here. The authors need to make a further effort to give the reader their interpretation of the results and their global impact/meaning. Among others, what is the meaning of the association to butyrate producers?

Response: We thank the Reviewer for this suggestion. We have now discussed the putative cross-feeding associated mechanistic basis of these links in our Discussions section (linking our observed association-patterns to the cross-feeding interactions reported in literature). We have also discussed how our machine-learning based identification of specific functional-groups associated with these functions further support this link. The relevant discussions section now reads as:

"Some of these associations have mechanistic bases. The positive associations of *B. adolescentis/longum* with butyrate-producing Firmicutes, as well as other *Coriobacteriaceae* members like *Collinsella* (also known producers of SCFAs)[11,12], could be due to specific cross-feeding interactions[13]. Bifidobacteria produce acetate and can degrade dietary and host-associated glycans into simple sugars (e.g. fucose, galactose), all of which can then internalized by these saccharolytic gut fermenters for their growth and as for precursors for SCFA production. SCFAs feed the human intestinal epithelial cells and stimulates production of mucins acting as sources of glycan fuelling Bifidobacteria growth thus resulting in symbiotic cross-feeding cycle[14]. Notably, many of these associated functions, e.g. those involved internalization/transport of sugars and involved in SCFAs and SCFA precursors synthesis, are also identified in our machine-learning based identification of functional groups associated with increased *B. adolescentis/longum* relative-abundances (**Figure S26**)."

Additionally, based on these cross-feeding interactions between *Bifidobacterium* and butyrate-producers, we have also tried to hypothesize the mechanistic basis of the negative links with specific *Bacteroides* members.

"Interestingly, taxa of *Bacteroidetes* lineage, e.g *B. thetaiotaomicron* and *P. copri*, also have similar metabolic activities in the gut as Bifidobacteria (acetate production, dietary/host glycan digestion into simple sugars), and thus may compete with Bifidobacteria for similar metabolic roles. This could partly explain the consistent negative associations observed between these taxa and certain Bifidobacteria in the adult gut microbiomes (from IndustrializedUrban to RuralTribal cohorts)."

Comment 7. 497-508. This is not a discussion, but rather an additional summary of a part of the study.

Response: We have now shortened this section to convey the implications of these findings. The section reads as:

"The key highlight of this study is the development of microbiome-level Receptive-Scores, that highlight the translational relevance of our findings. These scores provide a quantitative measure of the microbiome's capacity to support colonization and persistence of the administered Bifidobacteria. Using multiple complementary frameworks, we show the utility of Receptive-Scores in predicting post-intervention increase/colonization/persistence of the administered Bifidobacteria as well as for stratifying individuals in personalized probiotic interventions."

Comment 8. 509-515. Lack of discussion, see above. What are the possible mechanistic links mentioned?

Response: We have now completely re-written the Discussion section especially this part, integrating discussion of the consistently identified taxa-to-taxa association-patterns, ML-identified taxa-functional-groups and what is known in the literature about cross-feeding links (both supportive and contradictory). We also highlight detrimental functional features. This relevant discussion section as summarized in the previous two responses now reads as:

"In this regard, some of these associations have mechanistic bases, and similar mechanistic signals also emerge from our machine-learning–based analysis of 162 non-Bifidobacterial functional groups. The positive associations of *B. adolescentis/longum* with butyrate-producing *Firmicutes*, as well as other *Coriobacteriaceae* members like *Collinsella* (also known producers of SCFAs)[11,12], could be to specific cross-feeding interactions[13]. Gut colonizing Bifidobacteria produce acetate and can degrade dietary and host-associated glycans into simple sugars (e.g. fucose, galactose), all of which can then internalized by these saccharolytic gut fermenters for their growth and as for precursors for SCFA production. SCFAs feed the human intestinal epithelial cells and stimulate production of mucins acting as sources of glycan fuelling Bifidobacteria growth and resulting in symbiotic cross-feeding cycle[14]. Notably, many of these associated functions, e.g. those involved internalization/transport of sugars and involved in SCFAs and SCFA precursors synthesis, are also identified in our machine-learning based identification of functional groups associated with increased *B. adolescentis/longum* relative abundances (**Figure S26**).

Interestingly, taxa of *Bacteroidetes* lineage, e.g *B. thetaiotaomicron* and *P. copri*, also have similar metabolic activities in the gut as Bifidobacteria (acetate production, dietary/host glycan digestion into simple sugars), and thus may compete with Bifidobacteria for similar metabolic roles. This could partly explain the consistent negative associations observed between these taxa and certain Bifidobacteria in the adult gut microbiomes (from IndustrializedUrban to RuralTribal cohorts).

In terms of the *B. breve*-associated functional-groups, preclinical studies and human trials have also shown the administration of Bifidobacterial probiotics to be associated with lower TMA levels in the gut and serum and the abundances of TMA-producing pathobiont Firmicutes, like *Clostridium asparagiforme/hathewayi/citroniae*[7,8]. This is also observed in our identification of glycine-betaine transporters (negatively linked to *B. adolescentis* abundance), that serves as a precursor of trimethylamine (TMA) and its downstream metabolite trimethylamine-N-oxide (TMAO), consistently linked to diseases such as atherosclerosis, neuroinflammation, and colorectal cancer[9,10]. However, the mechanistic bases of such links still need to be investigated."

Minor points

Comment 9. Be consistent: taxa, traits, representations, features all wording to express mainly the same things, basically microbiome composition.

Response: We apologize for this. We have now retained the following wordings throughout the manuscript.

a. We always referred to the Non-Bifidobacteria as 'non-Bifidobacterial taxa' or 'non-Bifidobacterial members'.

b. We use the Bifidobacterial features when we collectively refer to the abundances of the eight *Bifidobacteria* and overall *Bifidobacterium* detection. While referring to specific *Bifidobacteria* species, we use the term abundances.

c. All 'representations' are modified to relative abundances.

d. Other terms used uniformly used throughout the manuscript as ‘Association-Scores’ and Receptive-Scores’.

Comment 10. 99-100. What do the authors mean here by “representation” of Bifidobacterium members?
Response: We have replaced all use of representation with relative abundances.

Comment 11. 103. I am not sure a question mark is needed here.
Response: We have now rectified this.

Comment 12. 115-120. Receptive score must be explained here in its biological and impact meaning to be clearly interpreted by the reader.
Response: We have now added this definition of Receptive-Score at this section:
“Receptive-Scores quantify how accommodating a given microbiome is to post-administration increase or persistence of a specific *Bifidobacterium*, given its baseline microbiome composition and host context (age group, life-style, disease status, and 16S or WGS sequencing type).”

Comment 13. 204-206. Unclear phrase.
Response: We have now rectified this.

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Response to reviewers

Reviewer #1

Comment 1. I have read the rebuttal of the manuscript entitled "Identifying the non-Bifidobacterial hallmarks of a Bifidobacterium-receptive gut microbiome" by Goswami et al. The authors have performed a great job in complementing the analysis by several key parameters such as confounders and quantitative approach for instance.

Response: We thank the reviewer for the positive comment.

Comment 2. The paper looks very heavy in figures (they are very dense). I would actually propose that Figure S1 is integrated as a main figure (Fig 1) and move one of the dense figures to supp info.

Response: We really thank the reviewer for this suggestion. We have now replaced the **Figure S1** (earlier) as **Figure 1** (new) and also replaced the **Figure 2** (the densest figure among the figures from the last submission) as **Figure S3**. Accordingly, we have re-numbered all the display items (Figures and Supplementary Figures) and modified their calling in the manuscript doc and wherever it's necessary.

Comment 3. The discussion can add a more specific section of the novelty in term of ecology beyond the receptive score

Response: We thank the reviewer for this insightful suggestion. In response, we have added a section to the Discussion highlighting the ecological novelty of our findings, as recommended by the reviewer. The revised text reads as follows-

"Beyond microbiome receptivity, our findings further provide insights into how the gut microbiome likely operates as a trophic network that is shaped by cross-feeding and metabolic dependencies, wherein cooperativity and competitive interactions shape structure community assembly and influence probiotic colonization outcomes. Our data identify taxa whose interactions can be further explored at functional resolution level in future studies that integrate multi-omics measurements, including metagenomics, metabolomics, flux-based modelling and artificial *in vitro* consortia. Controlled perturbation and longitudinal intervention studies can also help establish causal links between ecological network structure and probiotic persistence dynamics."

Reviewer #2

Comment 1. The authors have done a satisfactory job in addressing all the criticisms highlighted in the previous report.

Response: We sincerely thank the reviewer for the positive comments and the constructive suggestions during the revision phase that has helped us to substantially improve our study.