Title Page

The abundance of potential vitamin B_{12} producers in gut microbiome is inversely associated with serum vitamin B_{12} levels: A putative role of Host-microbiome feedback regulation

Nisha Chandel[1‡], Priyansh Patel[1‡], Pramod R. Somvanshi[1,2], Anil Kumar Verma[3], Vivek Thakur[1*]

[1]Department of Systems & Computational Biology, School of Life Sciences, University of Hyderabad, India

[2]Asian Centre for Endocrine Pancreas Research, Asian Healthcare Foundation, AIG Hospitals, Hyderabad, India

[3]ICMR-National Institute of Research in Tribal Health (NIRTH), Jabalpur, India

[‡] The authors have contributed equally to the work

* Corresponding author: vivek22@uohyd.ac.in

List of abbreviations

CNNS: Comprehensive National Nutrition Survey

IF: Intrinsic factor

MAGs: Metagenome-Assembled Genomes

MCM: Mitochondrial L-Methyl-malonyl-CoA mutase

MetH: Methionine synthase

NRC: Nutrition Rehabilitation Centre

RDI: Recommended dietary intake

Vit. B_{12}: Vitamin B_{12}

Abstract

Background: The human gut microbiome is a natural source of essential micronutrients like B-vitamins, which are utilized by both the host and other community members. The distribution of prevalence and abundance of known B-vitamin producers in Indian cohorts was reported to be marginally higher than that of the entire gut microbiome.

Objective: To test whether the presence of B-vitamin producers/biosynthetic pathways translates into serum B-vitamin levels, taking B_{12} as a case example.

Methods: Fecal samples were collected from non-deficient (n=29) and B_{12} deficient (n=30) children from a tribal region of central India. Whole metagenomic DNA was extracted, sequenced, and analyzed for taxonomic profiling and diversity comparisons. Differentially abundant taxa between two groups were identified. The prevalence and abundance of potential B_{12} producers were compared, and their association with serum B_{12} level was established.

Results: A comparison of *within-sample* diversity between the two groups didn't show any difference; however, *between-sample* diversity was significantly less in the B_{12} deficient group. Differential abundance testing also reflected changes in microbiome structure upon B_{12} deficiency, where an increased abundance of B_{12} transporter-carrying *Bacteroides thetaiotaomicron*, a few pathogenic species, and ten known B_{12} producers was observed in the deficient group. Potential B_{12} producers were also significantly prevalent and abundant in the deficient group. Their cumulative abundance was also significantly higher in the deficient group and showed a negative association with serum B_{12} levels.

Conclusion: B_{12} absorption in the colon supports the idea that gut microbes contribute to its supply. Higher potential B_{12} producers in the deficient group suggest an adaptive mechanism by the gut microbiome to meet the community's B_{12} requirements. Even at low bioavailability, the

gut microbiome appears to selectively promote the growth of B_{12} producers either due to selective pressure or due to the host's efforts to maintain B_{12} homeostasis, indicating host-microbiome feedback regulation.

Introduction

Cobalamin (Vitamin B_{12}) is one of the structurally most complex biomolecules, exclusively synthesized by bacteria and archaea [1, 2]. It is needed as a co-factor only for two enzymes in humans, namely, cytosolic Methionine synthase (MetH) and mitochondrial L-Methyl-malonyl-CoA mutase (MCM)[3]. Being an essential micronutrient, it must be acquired by ingestion [1]. Its deficiency inhibits purine synthesis, impairs DNA synthesis, and causes erythroblast apoptosis, resulting in anemia (reviewed by Koury and Ponka; 2004) [4]. B_{12} depleted diet, malabsorption, and defects in the cellular delivery and uptake system are the causes of deficiency, as reviewed in multiple studies [5-7]. The recommended dietary intake (RDI) of vitamin B_{12} (Vit. B_{12}) ranges from 0.9 to 2.4 micrograms from infants to adults, which can be sourced from an animal-based diet, fortified and biofortified foods [8]. Ingestion of such foods brings Vit. B_{12} to the small intestine, where it binds to an intrinsic factor (IF) released from the stomach. This IF-B_{12} complex gets absorbed in the small intestine. The unabsorbed B_{12} is utilized by bacteria in both the small and large intestine (see the review articles) [7, 9].

Biosynthesis of Vit. B_{12} is one of the essential functions of the gut bacterial community [10, 11]. However, approximately 25% of sequenced gut bacterial genomes had the genetic potential to synthesize corrinoids (cobalamin and related cofactors), whereas 80% were predicted to use

them [12], indicating that the majority of the community members depend on B_{12} bio-synthesizers and unabsorbed dietary B_{12} for their requirement.

Vit. B_{12} modulates the gut community structure, as it is best known for its role as a cofactor and gene expression regulator in bacteria [13]. Different gut bacterial species require distinct corrinoids, and they obtain specific ones by corrinoid remodelling, which limits the corrinoid availability to the host [13, 14]. Several possible competitive and symbiotic interactions within the gut community indicated the co-evolution of community members, which possibly has also benefited the vitamin homeostasis of the host [11].

Earlier, we reported the profiling of the biosynthetic potential of gut microbes for all B vitamins in Indian samples [10] and estimated a slightly higher contribution of such microbes to RDI than a previous report [11]. On the contrary, a contemporary country-wide nutritional survey (Comprehensive National Nutrition Survey, CNNS-2018) reported a worrying level of prevalence of Vit. B_{12} deficiency in the Indian population [15]. This made us wonder if i) a high abundance of B_{12} producers or B_{12} biosynthesis pathways in the gut gets translated into host serum B_{12} levels, and ii) serum B_{12} deficiency shows any association with the gut community structure. The current study aims to answer these questions by analyzing fecal metagenome data from B_{12} deficient (n=30) and non-deficient (n=29) children from the Balaghat district of Madhya Pradesh, India, where we assumed that fecal metagenome is a proxy of the gut metagenome and tested the association of host serum B_{12} level with potential gut B_{12} producers (Figure 1A).

Materials and Methods

Sample Collection:

Sampling sites included the Nutrition Rehabilitation Centre (NRC), Anganwadi centres (part of the Integrated Child Development Services program), and the Government Primary School of wards 3, 4, and 9 of Baihar village in the Balaghat district of Madhya Pradesh state, India. Children who hadn't taken antibiotics for the last month and didn't have diarrhoea, constipation, other gastrointestinal diseases, or any other disease were included in this study. Metadata about all subjects was gathered using a questionnaire and consent form, which was approved by the Institutional Ethics Committee (UH/IEC/2022/389), University of Hyderabad, India and ICMR-National Institute of Research in Tribal Health, India (Table S1). The fecal sample collection was done from January 2023 to March 2023, involving children with ages ranging between 2-12 years as follows: serum B_{12} profiling of 158 children was first done at Thyrocare Technologies Ltd. (Mumbai) using CMIA/CLIA methods, and they were classified into non-deficient (>210 pg/ml; n=118) and B_{12} deficient groups (<210 pg/ml; n=40). After obtaining 40 B_{12} deficient cases, further screening of serum B_{12} profiling was stopped as it was an exploratory study. The fecal samples, however, could be obtained only for 30 of them. To make both groups comparable, 29 non-deficient children were randomly selected for fecal sampling. About 30 participants per group is a common starting point in exploratory research due to its ability to balance statistical power with practical feasibility, its suitability for detecting small-to-medium effects in high-dimensional microbiome data, and its alignment with power analysis estimates for achieving a reasonable likelihood of detecting true effects in small-to-medium-sized studies. The fecal samples were collected in sterile stool containers and stored at 0°C within 1 to 5 hours and later were transported in dry ice to miBiome Therapeutics LLP Mumbai, India, for DNA extraction and sequencing (Figure 1A).

Fecal Metagenomic DNA extraction and quantification:

Whole metagenomic DNA from all 59 samples was isolated using the QIAamp PowerFecal Pro DNA Kit according to the manufacturer's instructions. The DNA samples were quantified on NanoDrop One and Qubit using water and a standard as a control, respectively. DNA quality was estimated by agarose gel electrophoresis.

Library Preparation and Sequencing:

Libraries were prepared using QIAseq® FX DNA library kit from Qiagen. Briefly, 150 ng of DNA was used for fragmentation and end repair, followed by adapter ligation. Excess adapters were removed using AMPure XP purification beads, followed by PCR enrichment of adapter-ligated DNA and clean-up of library products as instructed by the manufacturer. The cleaned libraries were quantitated on a Qubit fluorometer, and appropriate dilutions were loaded on HS D1000 screen tape to determine the size range of the fragments and the average library size. Eventually, paired-end sequencing, with reads of size 150 bases each, was done using the Illumina *Novaseq6000* platform.

Pre-processing of metagenomic reads

The quality of sequencing data was checked using FastQC v0.12.1 [16] and MultiQC v1.22.1 [17]. Since all reads had a *Phred*-score > 25, only ambiguous bases and adapters were removed using *fastp* [18]. Further, host contamination in the filtered reads was removed by aligning reads to the human genome (GRCh38) using Bowtie2 v2.5.4 [19], and unaligned reads were extracted using SAMtools v1.20 [20]. The processed reads were used for downstream analysis (Figure 1B).

Taxonomy profiling and species filtration

The compositional profiling of each sample was done using MetaPhlAn 4.0.6 (Metagenomic Phylogenetic Analysis) [21]. To further reduce the risk of contamination or alignment artifacts,

species with very low mean relative abundance (<= 0.001%) or prevalence (<= 15%) were removed (Figure 1B).

Diversity analysis

The filtered compositional profiles were used to measure alpha and beta diversity between the two groups. The alpha diversity (or within-sample diversity) was measured by using the *vegan* package [22] using multiple measures such as species richness (*specnumber()* function), Shannon index (*diversity()*), and Simpson index (*diversity()*). The beta diversity (or between-sample diversity) analysis was first performed using a dimensionality reduction approach: Principal Coordinate Analysis (PCoA). It was done using the *cmdscale()* function on Bray-Curtis distance matrix. Alternatively, the beta diversity analysis was performed using pair-wise distance measures like Bray-Curtis, Jaccard distance, and Robust Aitchison distances; all were calculated using the *adonis()* function in the *vegan* package in R.

Vit. B_{12} pathway analysis at the community (or microbiome) level

For Vit. B_{12} biosynthesis pathway analysis, filtered reads of samples from each group were co-assembled using the *de novo* assembly tool, MEGAHIT (v1.2.9) [23]. The contigs of each co-assembly were annotated using the Prokka tool (v1.14.5) [24]. Gene abundance in each sample was estimated by mapping filtered reads from the respective sample to the gene sequences of the corresponding co-assembly using the Bowtie2 tool (v2.5.4) [19], and the read counts were estimated by the Expectation-Maximization-based tool, RSEM (v1.3.3) [25] (Figure 1B).

To examine the presence of B_{12} biosynthesis pathway(s) in any sample, the reference genes of the known B_{12} biosynthesis pathways or subpathways in bacteria were first obtained from MetaCyc [26] (Table 1). Since the majority of the reference genes were from *Salmonella typhimurium* genome (RefSeq ID: GCF_000006945.2) [27], the genome annotation using Prokka

tool [24] was used to obtain the protein sequences. The reference genes which were from other bacteria, namely, *Listeria innocua serovar 6a* (strain ATCC BAA-680 / CLIP 11262), *Escherichia coli (strain K12)*, *Priestia megaterium*, *Desulfuromonas acetoxidans* (strain DSM 684 / 11070), *Eubacterium callanderi*, their protein sequences were obtained from UniProt database [28], and appended to *S. Typhimurium* protein sequences. The orthologs of the appended set were identified among translated gene sequences of each co-assembly using the OrthoFinder tool (v2.5.5) [29] (Figure 1B).

The pathway abundance in each sample was estimated by taking the median value of normalized abundances of all genes of the pathway. The abundance of any gene was independent of the genomes to which they belonged.

Vit. B_{12} pathway analysis at Metagenome-Assembled Genomes (MAGs) or species level

The above analysis was based on B_{12} biosynthesis through cooperativity at the community level. Additionally, the contribution from the individual MAGs with complete pathways was also considered for comparison between the groups. The contigs from each co-assembly were binned to obtain MAGs using the CONCOCT tool (v1.1.0) [30]. Their quality was assessed with CheckM2 tool (v1.0.2) [31], and MAGs with completeness <= 90 % were discarded. Complete MAGs were annotated using the Prokka tool (v1.14.5) [24], and their taxonomy was predicted using GTDB toolkit [32]. Orthologs of the B_{12} biosynthesis pathways gene in each MAG were identified by using the OrthoFinder tool (v2.5.5) [29] (Figure 1B). The mean contig depth (calculated by CONCOCT) belonging to a particular MAG was used as its abundance. The proportion of samples having a MAG was termed as its prevalence. Pathways with completeness <= 80% and present only in 5 MAGs or less than that were eliminated from the downstream analysis.

Differential abundance testing and other statistical Analyses

All statistical analyses were performed in R (version 4.2.2; www.r-project.org). The difference in beta diversity was statistically tested using the *adonis()* function in the vegan package, which uses PERMANOVA. Statistical testing for differentially abundant species was done using a negative binomial regression model-based *Wald* test implemented in DESeq2 [33] after controlling for the effect of confounders, namely batch, age, gender, diet, and BMI. The known B_{12} producers were inferred by comparing the differentially abundant species to a comprehensive list from our previous study [10].

All the statistical comparisons between groups were performed using the non-parametric (*Wilcoxon rank-sum)* test. The proportion of MAGs carrying B_{12} pathways (potential B_{12} producers) between the two groups was compared using the proportionality test function (*prop.test()*). All figures were generated using the *ggplot2* package [34] in R.

Results

The prevalence of B_{12} deficiency in the tribal children cohort was higher than the national/state average for school-age children

Serum B_{12} profiling of 158 children from the Balaghat district of central India showed a deficiency in ~25% of samples (Supplemental Table 2). Compared to CNNS-2018 survey data, though the prevalence in the current study had a similar increasing trend with age (10%, 28%, and 39.3%, in pre-school, school-age and adolescent, respectively), however, the prevalence in the school-aged group was relatively higher (~28%, n=90) (Supplemental Table 2).

From the total of 158 subjects, two groups of similar size were made (see methods) for further study: one with a normal B_{12} level (n=29, range: 212-810 pg/ml, median=365 pg/ml) and another

with B_{12} deficiency (n=30, range: 82-198 pg/ml, median=169 pg/ml) (Figure 1A, Supplemental Figure 1A and Supplemental Table 1). Whole metagenome shotgun sequencing of fecal samples of both the groups resulted in ~ 25 million paired-end reads per sample (~24 million after quality control) (Supplemental Figure 1B).

Mixed trend for change in alpha (within-sample) diversity in the B_{12} deficient group

Taxonomic profiling results showed the presence of 1225 species (Supplemental Table 3), where most of the species belonged to phylum Bacillota, Bacteroidota, Actinomycetota, and Pseudomonadota (Figure 2A). It was also observed that ~20% of the reads remained unclassified, which may be partly due to the uniqueness of the Indian gut microbiome (Figure 2A). Species present only in a few samples and with low abundance were filtered (see materials and methods), and the remaining 374 species were considered for downstream analysis. Within-sample diversity showed no significant change in the B_{12} deficient group when estimated using metrics based on both the richness and evenness, such as the Shannon index (p = 0.11) and Simpson index (p = 0.10). However, it was significantly higher when estimated using only the species richness (p = 0.05) (Figure 2B and C; Supplemental Figure 2A).

Significant reduction in beta (between-sample) diversity in the B_{12} deficient group indicated convergence

To examine if the microbiome composition changed upon B_{12} deficiency, PCoA using the Bray-Curtis dissimilarity index was done. B_{12} deficient samples clustered together, partially overlapping with the non-deficient ones along the first principal coordinate, which captured ~24% of the variation (Figure 3A). The higher similarity (i.e., lower distance) among the B_{12} deficient samples compared to non-deficient ones was statistically confirmed using the

PERMANOVA test (p = 0.004) (Figure 3B). Similar trends of lower beta diversity in B_{12} deficient group were observed using other distance measures like the Jaccard distance (p = 0.005) and the Robust Aitchison distance (p = 0.07), further confirming that gut community structure changed upon B_{12} deficiency (Figure 3C; Supplemental Figure 2B).

Ten *known* B_{12} producers were among sixty-two differentially more abundant species in the B_{12} deficient group

To find out differentially abundant species in any of the groups, their abundance was statistically compared. A total of 157 significantly abundant species, 62 were more abundant in the B_{12} deficient group (FDR = 0.05, $\log_2$ fold change >=3) (Figure 3D), which included the B_{12} transport system carrying species of genus *Bacteroides*, pathogenic species *Sutterella faecalis*, *Clostridiaceae unclassified SGB4771*, mucin degrader *Akkermansia muciniphila*, etc. [35 - 38]. The non-deficient group had a higher abundance of lactate metabolizing species of genus *Veillonella*, *Streptococcus thermophilus*, plant-based diet associate *Prevotella stercoria* (previously known as *P. copri*), a short-chain fatty acid (acetic acid) producer *Eubacterium siraeum*, probiotic potential candidate *Blautia* etc, [39 - 43]. This indicated gut community perturbation upon B_{12} deficiency, resulting in a higher abundance of at least a couple of pathogens and a lower abundance of a few beneficial bacteria (Figure 3D; Supplemental Table 4). Further, the known B_{12} producers among the differentially abundant species were examined: the B_{12} deficient group had about ten known B_{12} producers, while only six were observed in the non-deficient group (Figure 3D).

Cooperativity-based B_{12} biosynthesis pathway abundance showed no change in the B_{12} deficient group

Anticipating the incompleteness of the literature information on B_{12} producers, the pathway abundance was further inferred directly from the metagenomic data, and its distribution was compared between the two groups. The pathway abundance in each sample was estimated assuming a cooperative interaction among community members for its biosynthesis. The abundance of all five B_{12} biosynthesis pathways/subpathways (Supplemental Table 5) was found to be statistically similar ($p > 0.05$), indicating no difference between the groups for the given assumption (Figure 4A-C; Supplemental Figure 3A and B).

Potential B_{12} producers were generally more prevalent and abundant in the B_{12} deficient group

An alternative to the cooperativity-based B_{12} biosynthesis assumption would be considering the contribution from the MAGs with a complete B_{12} biosynthesis pathway (i.e., the potential B_{12} producers) for abundance estimation. Out of the total of 324 MAGs in the non-deficient group and 367 in the B_{12} deficient group, 135 and 161 were complete, respectively. While the number of MAGs having a particular B_{12} biosynthesis pathway varied between both groups, their proportion was statistically similar (Table 1). Among five pathways, anaerobic, GDP-I, and Cobinamide salvage were present in more than 5 MAGs, so only these were considered for further analysis. The prevalence of the potential B_{12} producers was significantly higher in the B_{12} deficient group for all three pathways; however, their abundance was significantly higher for two of the pathways, with the anaerobic pathway an exception ($p=0.3$) (Figure 4C–I).

The cumulative abundance of potential B_{12} producers increased with the B_{12} deficiency

Instead of considering the abundance of potential B_{12} producers for individual pathways, their cumulative abundance in each sample was calculated, and a group-wise comparison was made. The B_{12} deficient group had a much higher cumulative abundance ($p = 7.955e\text{-}09$) (Figure 5A). To further establish the association between serum B_{12} level and the cumulative abundance of B_{12} producers, linear regression analysis was carried out. Results showed a negative association, where with a decline in serum B_{12} levels, the abundance of B_{12} producers significantly increased ($p = <0.001$) (Figure 5B). Moreover, applying local regression (locally weighted scatterplot smoothing, LOWESS) gave a more refined trend where the cumulative abundance of potential B_{12} producers increased exponentially when the serum B_{12} level approached the deficient range (Supplemental Figure 4). The taxonomic classification showed that the majority of the B_{12} producers belonged to the phylum Bacillota, followed by Bacteroidota. 18.4% and 21.21% of them remained unclassified in B_{12} deficient and non-deficient groups, respectively (Supplemental Table 6, Supplemental Figure 5A and B).

Discussion

To the best of our knowledge, the current study is the first one to directly elucidate the association between serum B_{12} level and gut microbiome at an improved species-level resolution and provide a better understanding than the existing reports. There have been multiple studies in humans and mice to examine the association[44-51], but all of them except one were done using 16S rRNA amplicon data and had a resolution at the genus level. The exception was a study on Saudi Arabian females using shotgun metagenome sequencing data; however, their aim was

slightly different as they examined the association between Vit. B_{12} status and gut microbiota in the context of obesity [51].

No significant difference between the two groups was observed for multiple measures of alpha diversity, except for the species richness. However, all measures of beta diversity were statistically different between the groups. Literature evidence on the association of B_{12} status with gut microbial diversity is limited. Results from the murine model suggested that gut microbial diversity or composition remained unaffected with the Vit. B_{12} level in the diet [46]. A systematic review on Vit. B_{12} and gut microbiome summarized that the alpha and beta diversity increase with an increase in Vit. B_{12}, as reported in three *in-vivo* studies; however, findings from animal (n=8) and observational human studies (n=11) were heterogeneous [52]. Hence, the mixed trend observed in our study was yet another addition to the heterogeneous diversity trends from the past studies.

The change in microbiome structure was also supported by the differential abundance of a few species in either group (Supplemental Table 4). Along with the enrichment of pathogenic species, the deficiency also led to an increase in the abundance of a corrinoid transporter-carrying *Bacteroides thetaiotaomicron*, and several other potential B_{12} producers. A lower relative abundance of the genus *Bacteroides* was reported to be associated with high B_{12} intake in humans [45, 47], and with B_{12} supplementation in mice and in-vitro models [52]. However, this trend was conflicted by another study, which reported no such change in the abundance [44]. *B. thetaiotaomicron*, a mucin generalist, has a crucial role in health and disease pathogenesis [53], and is also known to have multiple copies of corrinoid transporter (BtuFBCD) in its genome, which gives an advantage to this bacterium in distinguishing B_{12} analogs produced by several other members [12, 14, 54]. The higher relative abundance of *B. thetaiotaomicron* in B_{12}

deficient groups suggests a scarcity of the B_{12} in the gut. Overall, a higher abundance of genus *Bacteroides* is a characteristic feature of a Vit. B_{12}-depleted microenvironment.

Besides, the taxonomic classification of B_{12} producers and their comparison with the available knowledge of producers in the current study also revealed several new species that have biosynthetic potential. For instance, MAGs belonging to species *A. muciniphila, Agathobacter faecis, Agathobacter rectalis, Alistipes senegalensis, Anaerobutyricum hallii, Veillonella atypica etc.*, had B_{12} biosynthesis potential (Supplemental Table 6).

Enrichment of known B_{12} producers and higher prevalence and abundance of potential B_{12} producers in the deficient group, as well as their negative association with serum B_{12} level, indicated that host B_{12} level is linked with the gut microbiome. This association could be a result of a feedback response, possibly originating from the host to the gut microbial community, so that it could source a fraction of the microbially synthesized B_{12} to meet its DRI and thereby partially prevent acute deficiency. Alternatively, the low B_{12} bioavailability could also impose a selection pressure on the microbial community to favor those that could grow by synthesizing their own B_{12} over those solely depending on the cobalamin analogs in the lumen.

The results indicate that the gut microbiome may possess buffering ability for homeostasis of some of the essential micronutrients, wherein certain species respond to the micronutrient deficiency in a threshold-dependent manner (at subnormal levels) and undergo microbiome remodelling with an increase in abundance of relevant species for its replenishment. A sustained microbial abundance, along with Vit. B_{12} deficiency of subnormal levels (~82 pg/ml) suggests a threshold-dependent switch-like response (Supplemental Figure 5) that takes care of severe deficiency; however, it is insensitive to achieve clinically sufficient Vit. B_{12} levels. Such a sub-optimal feedback response may be evolutionarily efficient in sparring raw material for *de-novo*

Vit. B_{12} biosynthesis under severe micronutrient deficiency in the long term. The structure of the emergent feedback loop can be illustrated as shown in Figure 6.

In a recent study, Kurpad et al. (2023) reported a late absorption phase of B_{12}, largely contributed by the absorption in the ascending colon. They reasoned that this late-phase colonic absorption could be sourced from the gut microbiome [55]. The present study showed the sensitivity of the gut microbiome to the serum B_{12} level, such that there was a spike in the abundance and prevalence of known or potential B_{12} producers when the serum level dropped below the threshold. This sub-optimal feedback response of the gut microbiome, possibly to maintain the B_{12} homeostasis, further supports the Kurpad et al. (2023) on the potential contribution of the colonic microbiome to daily B_{12} requirements.

In our past work, we highlighted the higher abundance of B-vitamin producers and biosynthesis pathways in tribal cohorts as a desirable feature and discussed possible links to different diets, limited access to antibiotics, etc. [10]. Similarly, Das et al. (2019) reported the Chinese population to have higher B-vitamin biosynthesis pathways compared to the other three nationalities [56]. Having observed higher B_{12} producers and biosynthesis pathways in the serum B_{12} deficient group, the previous findings should be re-interpreted based on the feedback model. In both previous reports, the B-vitamin status of subjects was unknown, and the groups with higher abundance suggest that the higher abundance could be due to dietary consumption of just adequate or sub-optimal levels of vitamins that are in a range where the switch of the feedback mechanism is active. This indicates that the vitamin levels are partly derived from dietary sources and partly from the microbiome in the healthy host.

Limitations and future directions

The results were based on a small sample size involving only children. The profiling of biochemical markers to determine the comprehensive health and nutritional status of an individual was not done, which restricted us from taking into account other confounding effects in the analysis. The microbial signatures identified in our study could be experimentally investigated in a longitudinal study on an animal model by administering B_{12} deficient and sufficient diets.

Conclusion

This is the first study to directly elucidate the association between B_{12} level and gut microbiome at the species or strain level. Low Vit. B_{12} bioavailability can lead to serum level deficiency. It induces convergence in gut community structure where mucin-degrading bacteria, a few other pathogens, and known B_{12} producers dominate. With a higher occurrence of potential B_{12} producers in the deficient group and their negative association with serum B_{12} level, we hypothesize that the gut microbiome responds to the deficiency by selectively promoting the growth of producers or the host itself, which stimulates the community to maintain B_{12} homeostasis; however, but with subnormal levels.

Acknowledgements

We would like to acknowledge the University of Hyderabad's Institution of Eminence (IoE) for funding our research and the Centre for Modelling, Simulations, and Design (CMSD) for allowing the use of their High-performance Computer facility. Dr Suyash Shrivastava, and Ms

Prathysha, Scientists/Project staff from ICMR-National Institute of Research in Tribal Health, Jabalpur, Madhya Pradesh, for preparing the questionnaire; Dr. Devaraj Parasannanavar, Scientist D, ICMR-National Institute of Health, Hyderabad, for his valuable suggestions for grouping samples based on clinical data.

Authors' contributions

1. Designed research: VT, AKV, NC; prepared questionnaire: AKV; collected samples and data: PP, AKV; performed data analysis: NC, VT; performed statistical analysis: NC, PR; Interpreted findings: NC, VT, PR; wrote manuscript: NC, PP, VT, PR; All authors have read and approved the final manuscript.

Data Availability

The raw data is publicly available at NCBI Sequencing Read Archive under Bioproject ID PRJNA1114651.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Funding

The project and manpower involved in this research were financially supported by funding from the University of Hyderabad-Institution of Eminence (grant number: UoH-IoE-RC2-21-023) from 30 Aug 2021 to 30th Aug 2024.

Reference

1. Roth, JR. Lawrence, JG. Rubenfield, M. Kieffer-Higgins, S. Church, GM. Characterization of the cobalamin (vitamin B_{12}) biosynthetic genes of Salmonella typhimurium. Journal of bacteriology. 1993 Jun;175(11):3303-16.
2. Roth, JR. Lawrence, JG. Bobik, TA. Cobalamin (coenzyme B_{12}): synthesis and biological significance. Annual review of microbiology. 1996 Oct;50(1):137-81.
3. Allen, RH. Stabler, SP. Savagem DG. Lindenbaum, J. Metabolic abnormalities in cobalamin (vitamin B_{12}) and folate deficiency. The FASEB journal. 1993 Nov;7(14):1344-53.
4. Koury, MJ. Ponka, P. New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. Annu. Rev. Nutr.. 2004 Jul 14;24(1):105-31.
5. Allen, LH. Causes of vitamin B_{12} and folate deficiency. Food Nutr Bull. 2008 Jun;29(2 Suppl):S20-34; discussion S35-7. doi: 10.1177/15648265080292S105. PMID: 18709879.
6. Green, R. Miller, JW. Vitamin B_{12} deficiency. InVitamins and hormones 2022 Jan 1 (Vol. 119, pp. 405-439). Academic Press.

7. Stabler, SP. Allen, RH. Vitamin B_{12} deficiency as a worldwide problem. Annu. Rev. Nutr.. 2004 Jul 14;24(1):299-326.
8. National Institute of Health; Vitamin B12–Deficiency Anemia https://www.nhlbi.nih.gov/health/anemia/vitamin-b12-deficiency-anemia Accessed 30 Oct 2024
9. Rowley, CA. Kendall, MM. To B_{12} or not to B_{12}: Five questions on the role of cobalamin in host-microbial interactions. PLoS pathogens. 2019 Jan 3;15(1):e1007479.
10. Chandel, N. Somvanshi, PR. Thakur, V. Characterisation of Indian gut microbiome for B-vitamin production and its comparison with Chinese cohort. British Journal of Nutrition. 2024 Feb;131(4):686-97.
11. Magnúsdóttir, S. Ravcheev, D. de Crécy-Lagard, V. Thiele, I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Frontiers in genetics. 2015 Apr 20;6:148.
12. Degnan, PH. Barry, NA. Mok, KC. Taga, ME. Goodman, AL. Human gut microbes use multiple transporters to distinguish vitamin B_{12} analogs and compete in the gut. Cell Host & Microbe. 2014 Jan 15;15(1):47-57.
13. Degnan, PH. Taga, ME. Goodman, AL. Vitamin B_{12} as a modulator of gut microbial ecology. Cell metabolism. 2014 Nov 4;20(5):769-78.
14. Allen, RH. Stabler, SP. Identification and quantitation of cobalamin and cobalamin analogues in human feces. The American journal of clinical nutrition. 2008 May 1;87(5):1324-35.

15. Comprehensive National Nutrition Survey (2016-2018) https://nhm.gov.in/WriteReadData/l892s/1405796031571201348.pdf Accessed 20 Dec 2024
16. Andrews, S. Babraham Bioinformatics-FastQC a Quality Control Tool for High Throughput Sequence Data. 2010 https://www.bioinformatics.babraham.ac.uk/projects/fastqc (accessed August 2024)
17. Ewels, P. Magnusson, M. Lundin, S. Käller, M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016 Oct 1;32(19):3047-8.
18. Chen, S. Zhou, Y. Chen, Y. Gu, J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018 Sep 1;34(17):i884-90.
19. Langmead, B. Salzberg, SL. Fast gapped-read alignment with Bowtie 2. Nature methods. 2012 Apr;9(4):357-9.
20. Li, H. Handsaker, B. Wysoker, A. Fennell, T. Ruan, J. Homer, N. et al. The sequence alignment/map format and SAMtools. bioinformatics. 2009 Aug 15;25(16):2078-9.
21. Blanco-Míguez, A. Beghini, F. Cumbo, F. McIver, LJ. Thompson, KN. Zolfo, M. et al. Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. Nature Biotechnology. 2023 Nov;41(11):1633-44.
22. Dixon, P. VEGAN, a package of R functions for community ecology. Journal of vegetation science. 2003 Dec;14(6):927-30.
23. Li, D. Luo, R. Liu, CM. Leung, CM. Ting, HF. Sadakane, K. et al. MEGAHIT v1. 0: a fast and scalable metagenome assembler driven by advanced methodologies and community practices. Methods. 2016 Jun 1;102:3-11.

24. Seemann, T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014 Jul 15;30(14):2068-9.
25. Li, B. Dewey, CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC bioinformatics. 2011 Dec;12:1-6.
26. Caspi, R. Billington, R. Ferrer, L. Foerster, H. Fulcher, CA. Keseler, IM. et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic acids research. 2016 Jan 4;44(D1):D471-80.
27. The National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/ Accessed 01 Aug 2024
28. Bairoch, A. Apweiler, R. Wu, CH. Barker, WC. Boeckmann, B. Ferro, S. et al. The universal protein resource (UniProt). Nucleic acids research. 2005 Jan 1;33(suppl_1):D154-9.
29. Emms, DM. Kelly, S. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome biology. 2019 Dec;20:1-4.
30. Alneberg, J. Bjarnason, BS. de Bruijn, I. Schirmer, M. Quick, J. Ijaz, UZ. et al. CONCOCT: clustering contigs on coverage and composition. arXiv preprint arXiv:1312.4038. 2013 Dec 14.
31. Chklovski, A. Parks, DH. Woodcroft, BJ. Tyson, GW. CheckM2: a rapid, scalable and accurate tool for assessing microbial genome quality using machine learning. Nature Methods. 2023 Aug;20(8):1203-12.
32. Chaumeil, PA. Mussig, AJ. Hugenholtz, P. Parks, DH. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database.

33. Love, MI. Huber, W. Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology. 2014 Dec;15:1-21.

34. Wexler, AG. Schofield, WB. Degnan, PH. Folta-Stogniew, E. Barry, NA. Goodman, AL. Human gut Bacteroides capture vitamin B12 via cell surface-exposed lipoproteins. Elife. 2018 Sep 18;7:e37138.

35. Hiippala, K. Kainulainen, V. Kalliomäki, M. Arkkila, P. Satokari, R. Mucosal prevalence and interactions with the epithelium indicate commensalism of Sutterella spp. Frontiers in microbiology. 2016 Oct 26;7:1706.

36. Muñiz Pedrogo, DA. Chen, J. Hillmann, B. Jeraldo, P. Al-Ghalith, G. Taneja, V. et al. An increased abundance of Clostridiaceae characterizes arthritis in inflammatory bowel disease and rheumatoid arthritis: a cross-sectional study. Inflammatory bowel diseases. 2019 Apr 11;25(5):902-13.

37. Derrien, M. Collado, MC. Ben-Amor, K. Salminen, S. de Vos, WM. The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract. Applied and environmental microbiology. 2008 Mar 1;74(5):1646-8.

38. Scheiman, J. Luber, JM. Chavkin, TA. MacDonald, T. Tung, A. Pham, LD. et al. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. Nature medicine. 2019 Jul;25(7):1104-9.

39. Huang, YY. Lu, YH. Liu, XT. Wu, WT. Li, WQ. Lai, SQ. et al. Metabolic properties, functional characteristics, and practical application of Streptococcus thermophilus. Food Reviews International. 2024 Feb 17;40(2):792-813.

40. Precup, G. Vodnar, DC. Gut Prevotella as a possible biomarker of diet and its eubiotic versus dysbiotic roles: a comprehensive literature review. British Journal of Nutrition. 2019 Jul;122(2):131-40.

41. Moore, WC. Johnson, JL. Holdeman, LV. Emendation of Bacteroidaceae and Butyrivibrio and descriptions of Desulfomonas gen. nov. and ten new species in the genera Desulfomonas, Butyrivibrio, Eubacterium, Clostridium, and Ruminococcus. International journal of systematic and evolutionary microbiology. 1976 Apr;26(2):238-52.

42. Liu, X. Mao, B. Gu, J. Wu, J. Cui, S. Wang, G. et al. Blautia—a new functional genus with potential probiotic properties?. Gut microbes. 2021 Jan 1;13(1):1875796.

43. Valentini, L. Pinto, A. Bourdel-Marchasson, I. Ostan, R. Brigidi, P. Turroni, S. et al. Impact of personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota–The “RISTOMED project”: Randomized controlled trial in healthy older people. Clinical nutrition. 2015 Aug 1;34(4):593-602.

44. Boran, P. Baris, HE. Kepenekli, E. Erzik, C. Soysal, A. Dinh, DM. The impact of vitamin B12 deficiency on infant gut microbiota. European journal of pediatrics. 2020 Mar;179:385-93.

45. Gurwara, S. Ajami, NJ. Jang, A. Hessel, FC. Chen, L. Plew, S. et al. Dietary nutrients involved in one-carbon metabolism and colonic mucosa-associated gut microbiome in individuals with an endoscopically normal colon. Nutrients. 2019 Mar 13;11(3):613.

46. Lurz, E. Horne, RG. Määttänen, P. Wu, RY. Botts, SR. Li, B. et al. Vitamin B12 deficiency alters the gut microbiota in a murine model of colitis. Frontiers in nutrition. 2020 Jun 5;7:83.

47. Carrothers, JM. York, MA. Brooker, SL. Lackey, KA. Williams, JE. Shafii, B. et al. Fecal microbial community structure is stable over time and related to variation in macronutrient and micronutrient intakes in lactating women. The Journal of nutrition. 2015 Oct 1;145(10):2379-88.

48. Forgie, AJ. Pepin, DM. Ju, T. Tollenaar, S. Sergi, CM. Gruenheid, S. Willing, BP. Over supplementation with vitamin B_{12} alters microbe-host interactions in the gut leading to accelerated Citrobacter rodentium colonization and pathogenesis in mice. Microbiome. 2023 Feb 3;11(1):21.

49. Babakobi, MD. Reshef, L. Gihaz, S. Belgorodsky, B. Fishman, A. Bujanover, Y. Effect of maternal diet and milk lipid composition on the infant gut and maternal milk microbiomes. Nutrients 2020; 12: 2539 [Internet].

50. Selma-Royo, M. García-Mantrana, I. Calatayud, M. Parra-Llorca, A. Martínez-Costa, C. Collado, MC. Maternal diet during pregnancy and intestinal markers are associated with early gut microbiota. European journal of nutrition. 2021 Apr;60:1429-42.

51. Al-Musharaf, S. Aljuraiban, GS. Al-Ajllan, L. Al-Khaldi, N. Aljazairy, EA. Hussain, SD et al. Vitamin B12 status and gut microbiota among Saudi females with obesity. Foods. 2022 Dec 11;11(24):4007.

52. Guetterman, HM. Huey, SL. Knight, R Fox, AM. Mehta, S. Finkelstein, JL. Vitamin B-12 and the gastrointestinal microbiome: a systematic review. Advances in Nutrition. 2022 Mar 1;13(2):530-58.

53. Wang, C. Zhao, J. Zhang, H. Lee, YK. Zhai, Q. Chen, W. Roles of intestinal bacteroides in human health and diseases. Critical reviews in food science and nutrition. 2021 Nov 25;61(21):3518-36.

54. Desai, MS. Seekatz, AM. Koropatkin, NM. Kamada, N. Hickey, CA. Wolter, M. et al. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. Cell. 2016 Nov 17;167(5):1339-53.

55. Kurpad, AV. Pasanna, RM. Hegde, SG. Patil, M. Mukhopadhyay, A. Sachdev, HS. et al. Bioavailability and daily requirement of vitamin B12 in adult humans: an observational study of its colonic absorption and daily excretion as measured by [13C]-cyanocobalamin kinetics. The American Journal of Clinical Nutrition. 2023 Dec 1;118(6):1214-23.

56. Das, P. Babaei, P. Nielsen, J. Metagenomic analysis of microbe-mediated vitamin metabolism in the human gut microbiome. BMC genomics. 2019 Dec;20:1-1.

Table 1. List of the B12 biosynthesis pathways or sub-pathways examined in this study, number of MAGs from each group having one of the pathways, and *p-value* of comparison of their proportion between groups.

Pathway/Subpathway name	**Short name**	**MetaCyc ID**	**Non-deficient**	**B_{12} Deficient**	***p-value***
Adenosylcobalamin biosynthesis (anaerobic)	anaerobic	PWY-5507	27	46	*0.11*
AdoCbl biosynthesis from adenosylcobinamide-GDP I	GDP-1	PWY-5509	63	85	*0.3505*
AdoCbl biosynthesis from adenosylcobinamide-GDP II	GDP-II	PWY-7975	1	1	_
AdoCbl salvage from cobalamin	Cobalamin salvage	PWY-6268	6	5	*1*
Superpathway of AdoCbl salvage from cobinamide	Cobinamide salvage	COBALSYN-PWY	44	67	*0.139*
Total MAGs			**135**	**161**	

Main Figures

Figure 1. Methodology. **A** sampling and **B** computational analysis. BioRender software was used to create the figure under an academic license.

Figure 2. Alpha (within-sample) diversity trends. **A** Bar plot showing the relative abundance of detected phyla in the data. The Box plots represent within-sample diversity between Non-deficient and B_{12} deficient groups: **B** Richness (*pvalue: 0.05*), and **C** Shannon diversity (*pvalue: 0.11*).

Figure 3. Beta (between-sample) diversity trends. **A** Principal coordinate analysis of Bray-curtis distances, **B** Bray-curtis dissimilarity index (*p-value= 0.004*), **C** Jaccard distance (*p-value=0.005*), and **D** Volcano plot showing differentially abundant species between non-deficient and B_{12} deficient groups (*adj-p-value <= 0.05*). Differentially abundant known B_{12} producers have been highlighted.

Figure 4. B_{12} Biosynthesis pathway abundance in two groups. **A** anaerobic (*p-value= 0.48*), **B** GDP-I (*p-value= 0.09*), **C** Cobinamide salvage (*p-value= 0.32*).

Abundance and Prevalence of potential B_{12} producers in two groups. **D** anaerobic pathway abundance (*p-value= 0.303*), **E** prevalence (*p-value= 0.007*), **F** GDP-I pathway abundance (*p-value= 0.01*), **G** prevalence (*p-value= 1.246e-06*), **H** Cobinamide salvage abundance (*p-value= 0.03*) , **I** prevalence (*p-value= 2.817e-06*).

Figure 5. A Cumulative abundance of potential B_{12} producers between two groups, **B** Relationship between serum B_{12} level and cumulative abundance of B_{12} producers.

Figure 6 The putative Host-microbiome feedback regulation loop **A.** In case of sufficient Vit. B_{12} bioavailability, the serum Vit. B_{12} is maintained above the deficiency threshold, wherein the microbiome shares the Vit. B_{12} from the host with a relatively lesser contribution towards *de-novo* Vit. B_{12} synthesis due to putative inhibitory effect of serum Vit. B_{12} levels on microbiome B_{12} synthesis. **B.** In the case of insufficient Vit. B_{12} uptake or transport (due to lack of intrinsic factor), serum Vit. B_{12} levels fall below a threshold that leads to microbiome remodelling to enhance the abundance of Vit. B_{12} producers and the inhibitory effect on Vit. B_{12} synthesis is relieved so that it is sourced from the microbiome origin to stall the severe Vit. B_{12} deficiency.

Supplementary Tables

Supplemental Table 1. Metadata of samples selected for metagenomic analysis.

Supplemental Table 2. Age-wise prevalence of B_{12} deficiency in tribal children from Balaghat.

Supplemental Table 3. Taxonomic profile of all samples.

Supplemental Table 4. Differentially abundant species in B_{12} deficient group with respect to the non-deficient group.

Supplemental Table 5. Genes involved in various pathways and subpathways of cobalamin biosynthesis.

Supplemental Table 6. Taxonomic classification of vitamin B_{12} pathway carrying MAGs identified in both the groups.

Supplemental Figure 1. Summary of sampled B_{12} level and fecal microbiome sequencing data. **A** Distribution of serum B_{12} levels in Non-deficient and B_{12} deficient groups. **B** Distribution of average reads per sample before and after filtering.

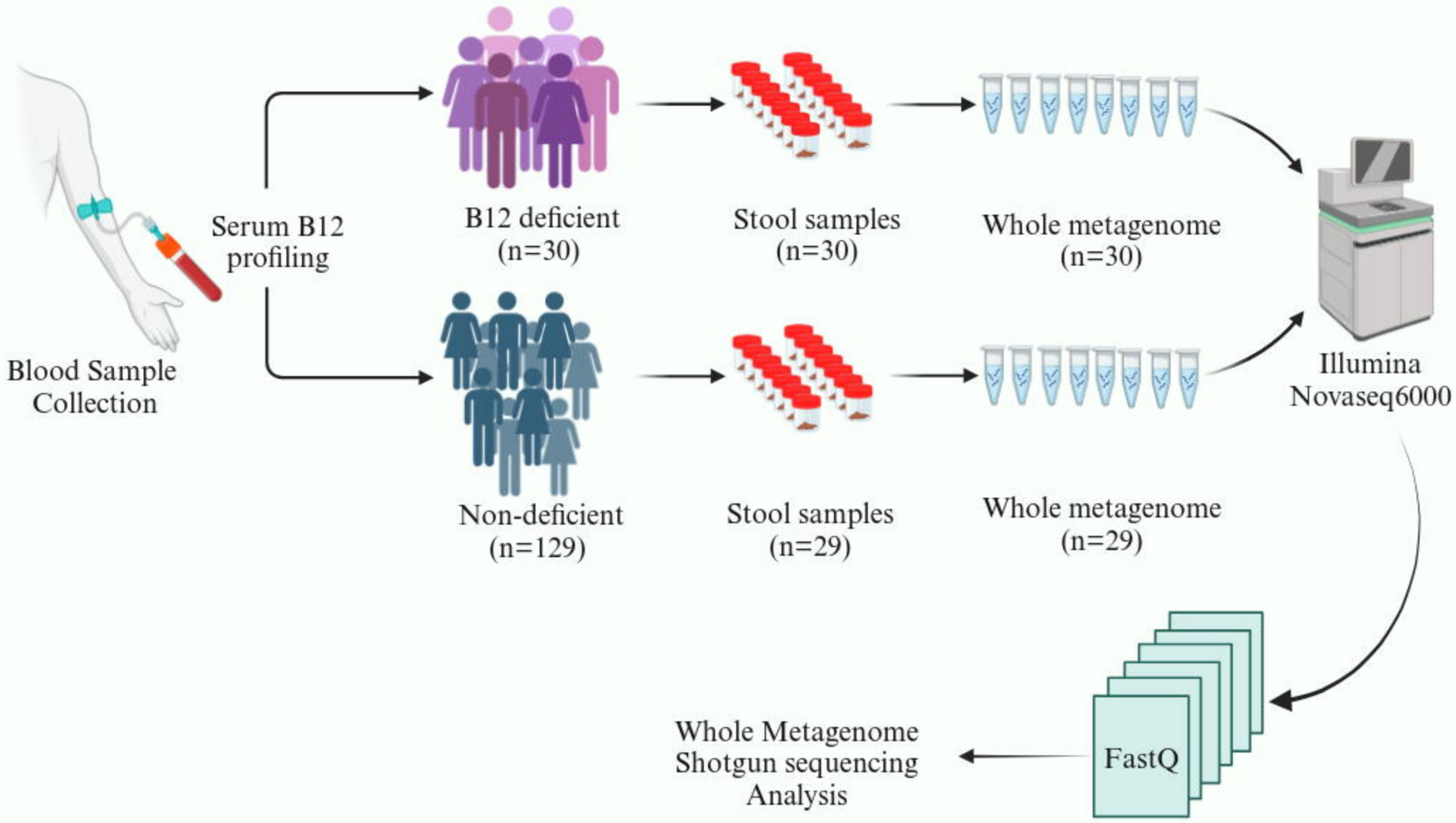
Supplemental Figure 2. Diversity trends. The Box plots represent diversity between Non-deficient and B_{12} deficient groups: **A** Simpson diversity (*p-value: 0.10*), and **B** Robust aitchison (*p-value= 0.07*).

Supplemental Figure 3. B_{12} Biosynthesis pathway abundance in two groups. A GDP-II (*p-value= 0.47*), **B** Cobalamin salvage *(p-value= 0.84)*

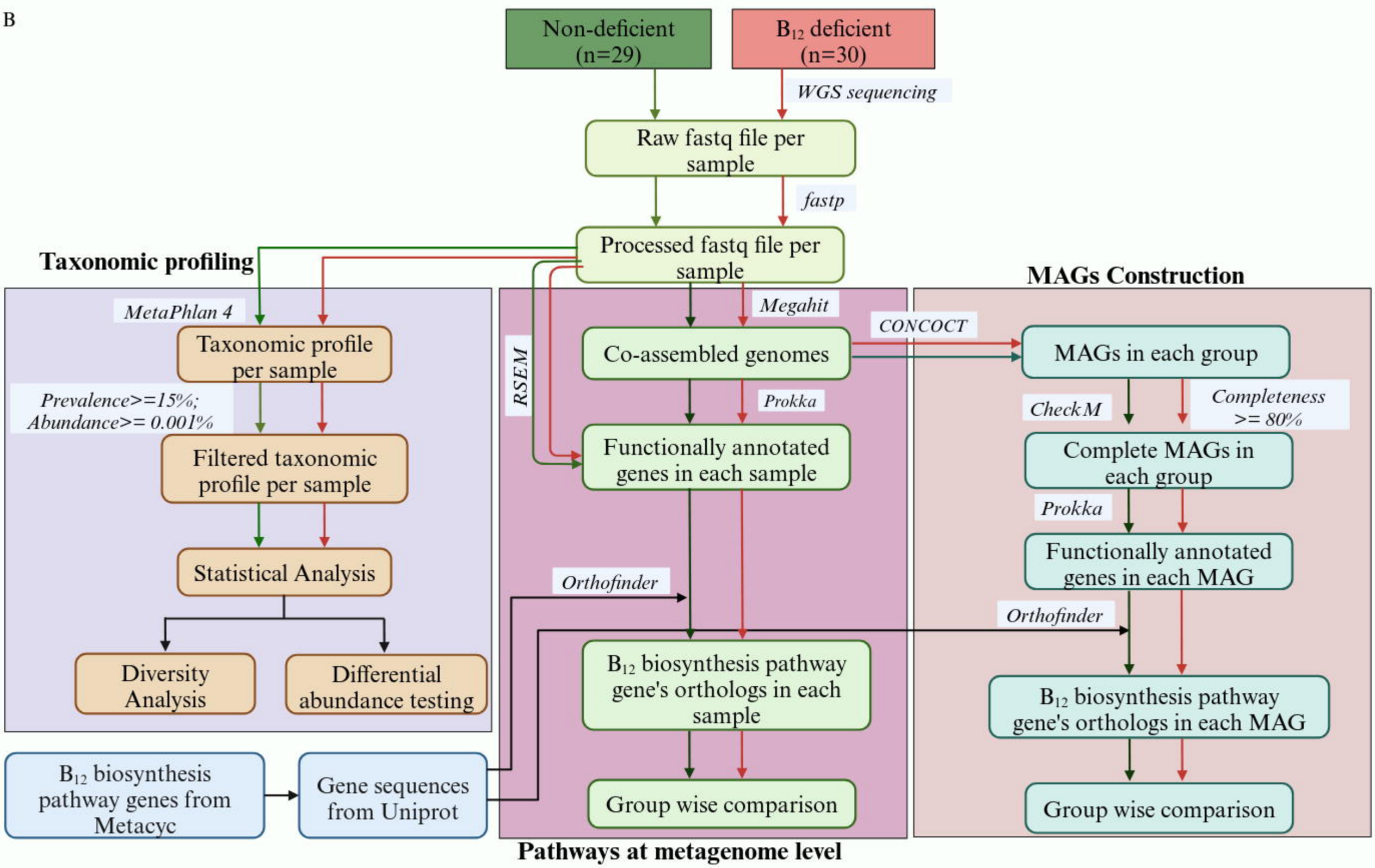
Supplemental Figure 4. Locally weighted scatterplot smoothing (LOWESS) of data in Figure 5B showed that the cumulative abundance of potential B_{12} producers increased exponentially when the serum B12 level lies in the deficient range.

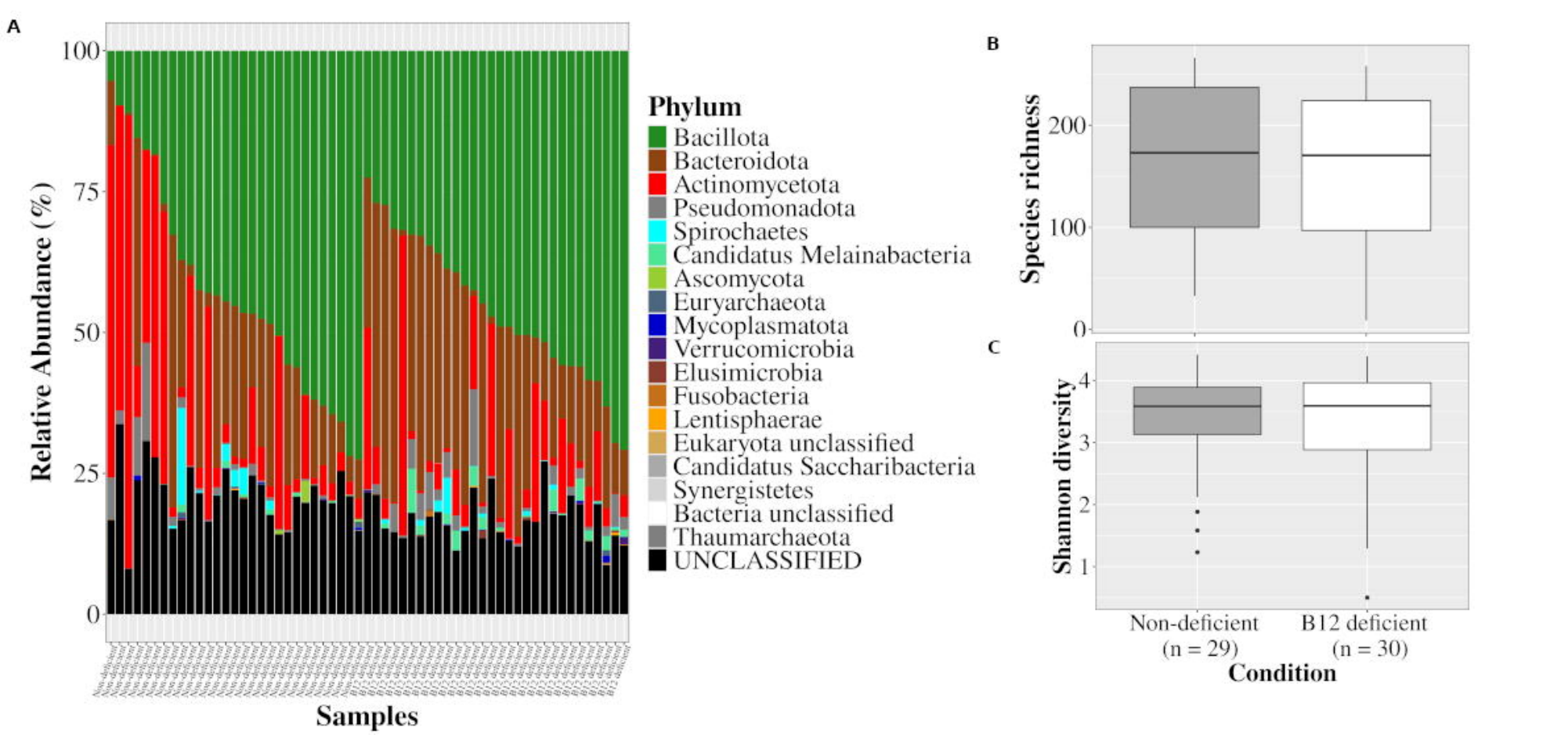
Supplemental Figure 5. Taxonomic classification of potential B_{12} producers at the phylum level in: A Non-deficient group, **B** B_{12} deficient group.

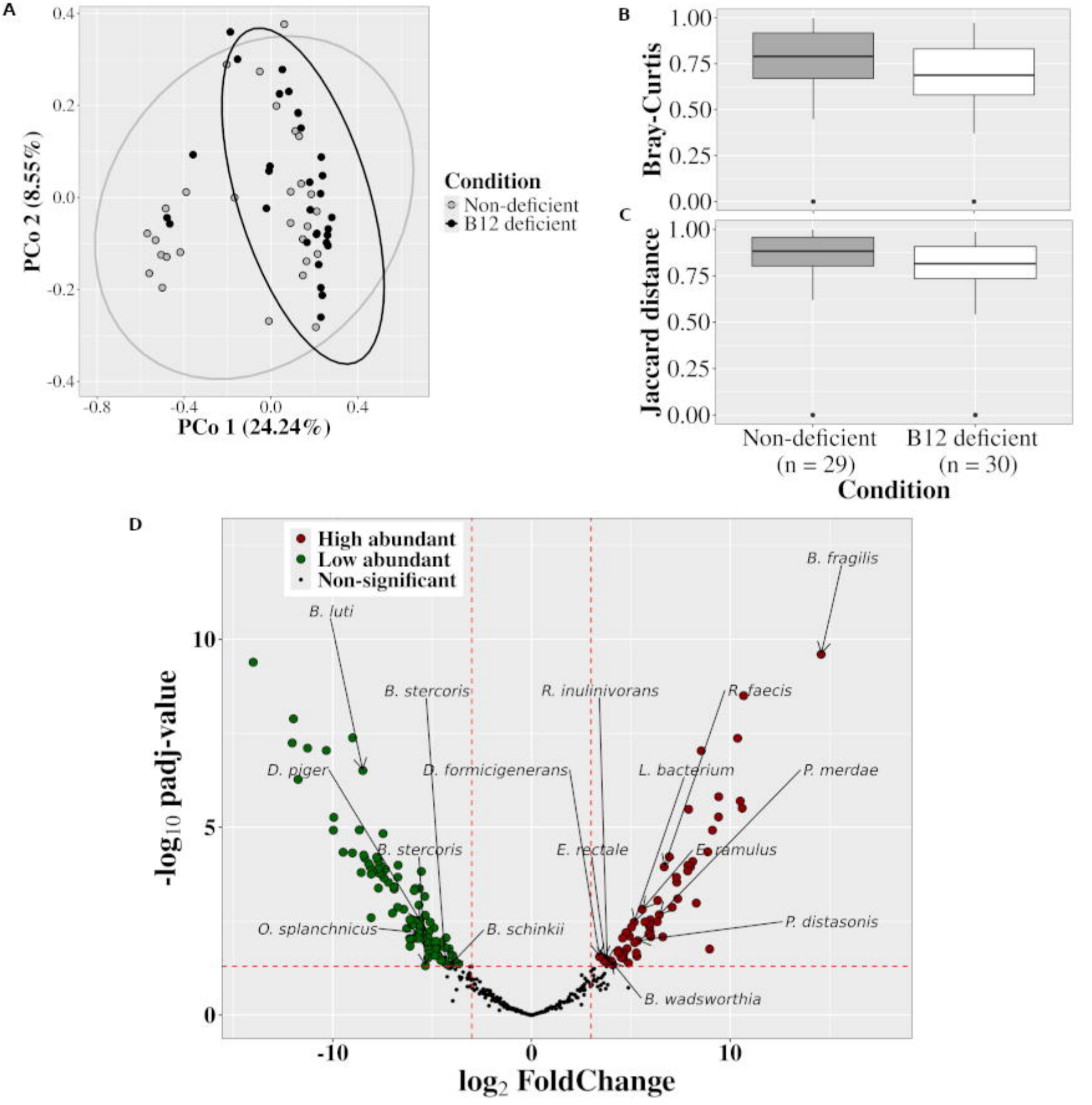
A

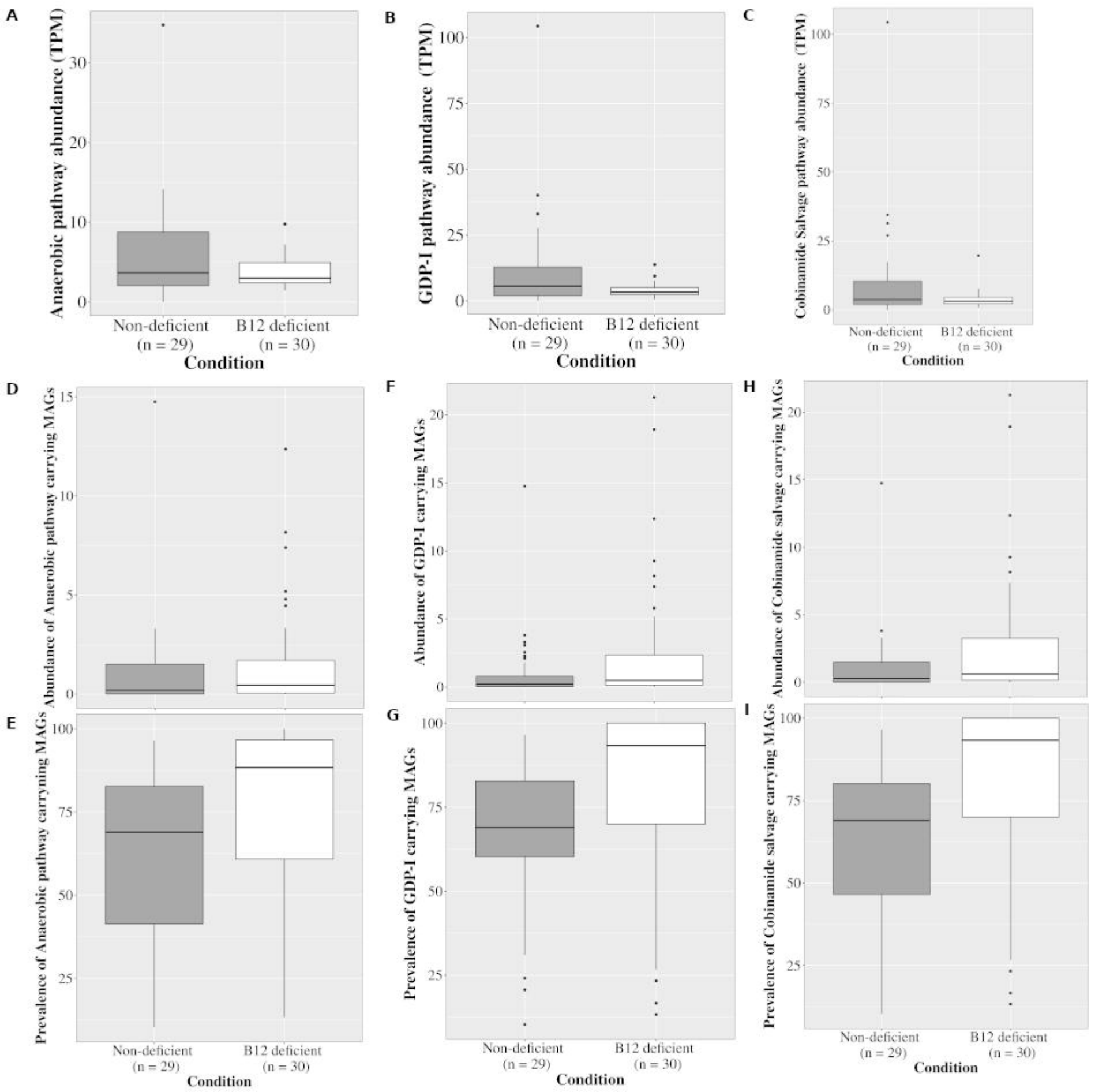

Blood Sample Collection
Serum B12 profiling
B12 deficient (n=30)
Non-deficient (n=129)
Stool samples (n=30)
Stool samples (n=29)
Whole metagenome (n=30)
Whole metagenome (n=29)
Illumina Novaseq6000
FastQ
Whole Metagenome Shotgun sequencing Analysis

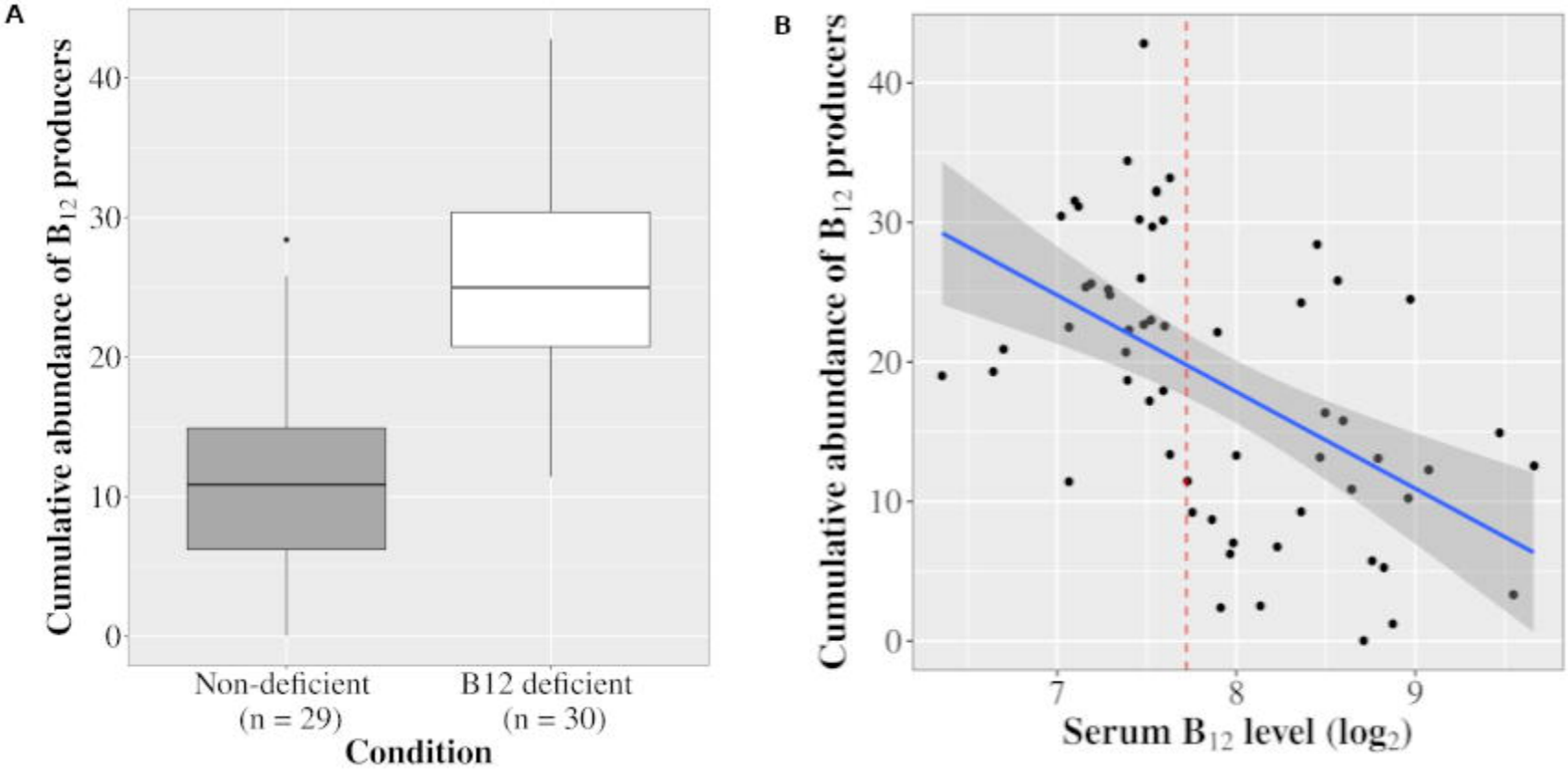
B


Non-deficient (n=29)
B12 deficient (n=30)
WGS sequencing
Raw fastq file per sample
fastp
Processed fastq file per sample
Taxonomic profiling
MetaPhlan 4
Taxonomic profile per sample
Prevalence>=15%; Abundance>= 0.001%
Filtered taxonomic profile per sample
Statistical Analysis
Diversity Analysis
Differential abundance testing
Megahit
RSEM
Co-assembled genomes
Prokka
Functionally annotated genes in each sample
Orthofinder
B12 biosynthesis pathway gene's orthologs in each sample
Group wise comparison
CONCOCT
MAGs Construction
MAGs in each group
CheckM
Completeness >= 80%
Complete MAGs in each group
Prokka
Functionally annotated genes in each MAG
Orthofinder
B12 biosynthesis pathway gene's orthologs in each MAG
Group wise comparison
B12 biosynthesis pathway genes from Metacyc
Gene sequences from Uniprot
Pathways at metagenome level


A
100
75
50
25
0
Relative Abundance (%)
Samples
Phylum
Bacillota
Bacteroidota
Actinomycetota
Pseudomonadota
Spirochaetes
Candidatus Melainabacteria
Ascomycota
Euryarchaeota
Mycoplasmatota
Verrucomicrobia
Elusimicrobia
Fusobacteria
Lentisphaerae
Eukaryota unclassified
Candidatus Saccharibacteria
Synergistetes
Bacteria unclassified
Thaumarchaeota
UNCLASSIFIED
B
Species richness
200
100
0
C
Shannon diversity
4
3
2
1
Non-deficient
(n = 29)
B12 deficient
(n = 30)
Condition

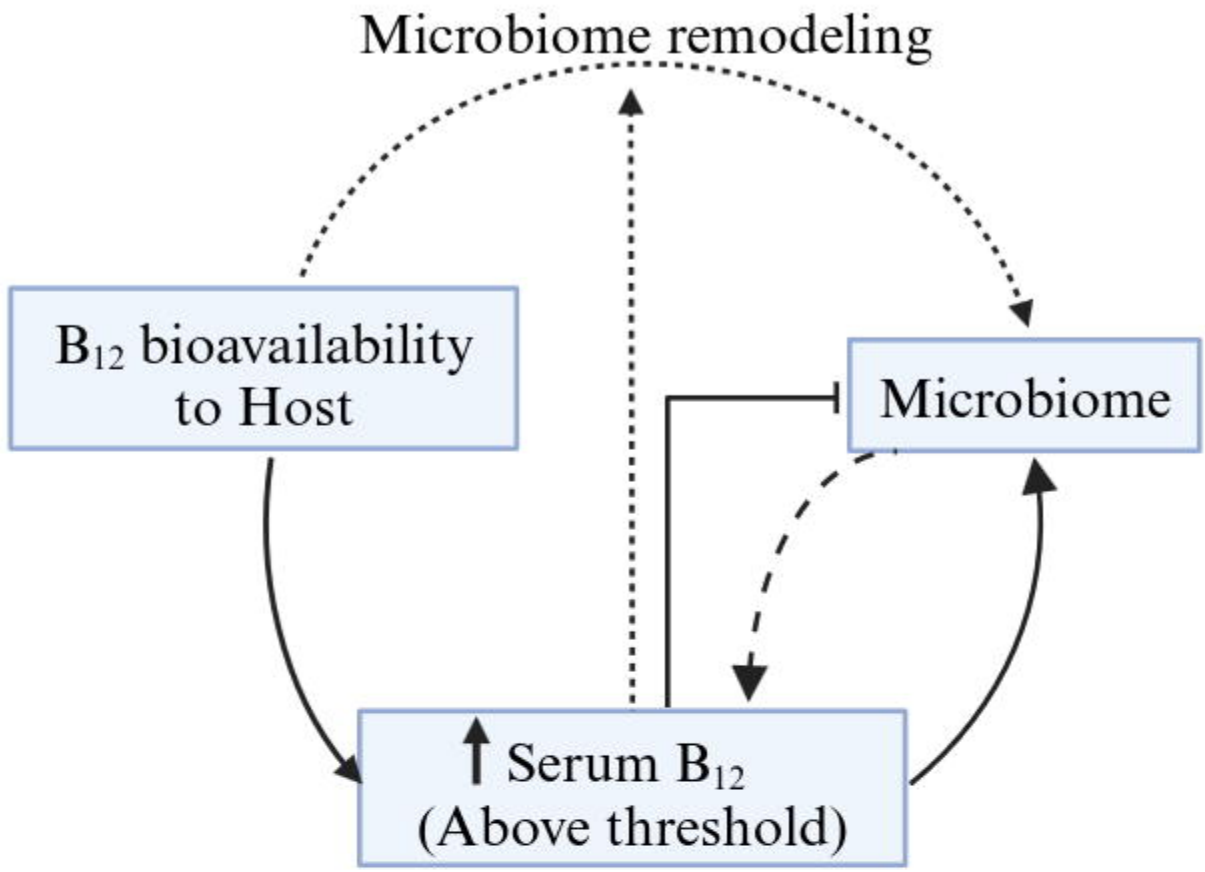

A
PCo 2 (8.55%)
PCo 1 (24.24%)
Condition
Non-deficient
B12 deficient
B
Bray-Curtis
C
Jaccard distance
Non-deficient
(n = 29)
B12 deficient
(n = 30)
Condition
D
High abundant
Low abundant
Non-significant
B. fragilis
B. luti
B. stercoris
R. inulinivorans
R. faecis
D. piger
D. formicigenerans
L. bacterium
P. merdae
B. stercoris
E. rectale
E. ramulus
O. splanchnicus
B. schinkii
P. distasonis
B. wadsworthia
-log10 padj-value
log2 FoldChange

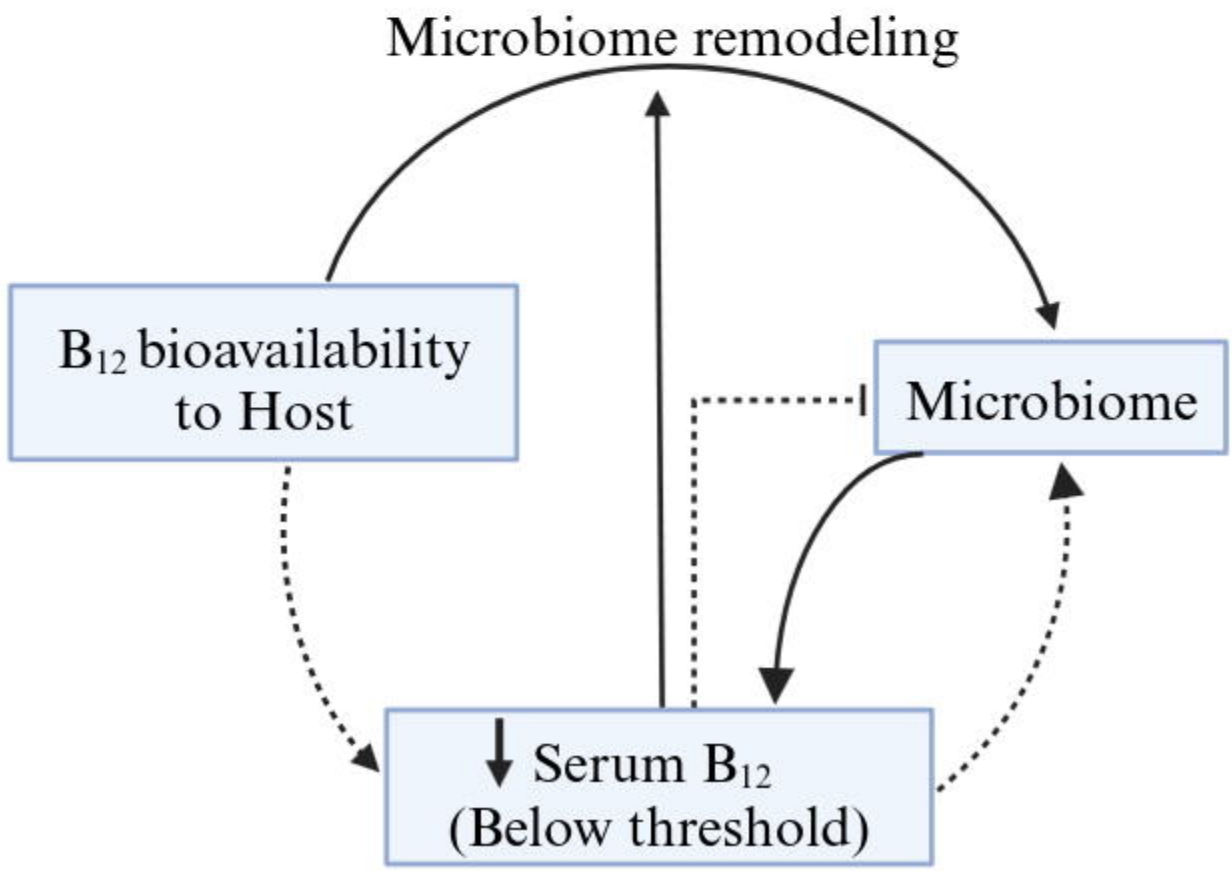

A
Anaerobic pathway abundance (TPM)
0
10
20
30
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
B
GDP-I pathway abundance (TPM)
0
25
50
75
100
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
C
Cobinamide Salvage pathway abundance (TPM)
0
25
50
75
100
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
D
Abundance of Anaerobic pathway carrying MAGs
0
5
10
15
E
Prevalence of Anaerobic pathway carrying MAGs
25
50
75
100
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
F
Abundance of GDP-I carrying MAGs
0
5
10
15
20
G
Prevalence of GDP-I carrying MAGs
25
50
75
100
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
H
Abundance of Cobinamide salvage carrying MAGs
0
5
10
15
20
I
Prevalence of Cobinamide salvage carrying MAGs
25
50
75
100
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition


A
Cumulative abundance of B_{12} producers
0
10
20
30
40
Non-deficient
(n = 29)
B12 deficient
(n = 30)
Condition
B
Cumulative abundance of B_{12} producers
0
10
20
30
40
7
8
9
Serum B_{12} level (log_2)

A

High/sufficient vitamin B_{12} bioavailability



B

Low/insufficient vitamin B_{12} bioavailability