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Inverse association between serum vitamin B_{12} level and abundance of potential B_{12}-producing gut microbes in Indian children

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List of abbreviations

p-adj: Adjusted p-value

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 prevalence and abundance of known B-vitamin producers and B-vitamin biosynthesis pathways have already been reported in gut microbiome cohorts of a few countries including India.

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 (serum B_{12} level > 210 pg/mL, n=29) and B_{12} deficient (serum B_{12} level < 210 pg/mL, 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 showed different microbiome structure in the B_{12} deficient group, where an increased abundance of B_{12} transporter-carrying *Bacteroides thetaiotaomicron*, a few pathogenic species, and ten known B_{12} producers was observed. 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: A higher abundance of potential B_{12} producers in the deficient group suggested an adaptive mechanism by the gut microbiome to meet the community's B_{12} requirements, by selectively promoting the growth of B_{12} producers.

Introduction

Cobalamin (Vitamin B_{12}) is one of the structurally most complex biomolecules, exclusively synthesized by bacteria and a few 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 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, and fortified 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} producers 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 remodeling, which limits their 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 biosynthetic potential of gut microbes for all B vitamins in Indian cohorts [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 such as age, gender, BMI, diet, and antibiotics or supplements usage, 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 basic summary of the data for the two groups is provided in Table 2. 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 Chemi Luminescent Microparticle Intrinsic factor Immunoassay (CMIA; Intra assay (%CV): 5.6, Inter assay (%CV): 6.8, Sensitivity: 125 pg/mL) or Competitive Chemi Luminescent Immuno Assay (CLIA; Intra assay (%CV): 5.0%, Inter assay (%CV): 9.2 %; Sensitivity: 45 pg/ml) 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 an average *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, which uses Benjamini-Hochberg procedure (FDR) for multiple hypothesis testing. The adjusted *p-value (p-adj) <= 0.05* was considered significant. 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()*). The association between serum B_{12} levels and the cumulative abundance of B_{12} producers was assessed using a multiple linear regression model in R (*lm()*), controlling for

potential confounders such as age, gender, diet, and BMI. 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; Table 2; 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 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 difference 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 was different in B_{12}-deficient condition, 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 the 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 different gut community structure in B_{12} deficient condition (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*, and others, which are listed in Supplemental Table 4 [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* and others (Supplemental Table 4) [39 - 43]. This indicated gut community perturbation in B_{12} deficient condition, where a higher abundance of at least a couple of pathogens and a lower abundance of a few beneficial bacteria (Figure 3D) was observed. 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 difference 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 was higher in the B_{12} deficient group

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, a multiple 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 ($p = <0.001, \beta_0 = 6.42, \beta_1 = -0.02$, *Std. Error = 0.006*) (Figure 5B).

Moreover, applying local regression (locally weighted scatterplot smoothing, LOWESS) revealed a refined trend, suggesting that the cumulative abundance of potential B_{12} producers increased non-linearly as the serum B_{12} levels approached the deficient range (Supplemental Figure 4). 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 association0 [44-52], 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].

In our study, we observed no significant differences in alpha diversity between the two groups, while a lower beta diversity was noted in the Vit. B_{12} deficient condition. These findings were partially consistent with previous reports. However, the literature evidence on the association between Vit. B_{12} status and gut microbial diversity remains limited. A systematic review on Vit. B_{12} and the gut microbiome summarized that both alpha and beta diversity increased with an increase in Vit. B_{12} levels, as reported in *in-vivo* studies; however, findings from mouse models

and observational human studies were heterogeneous [53]. Two of the mouse studies did not show any difference in alpha diversity [46,54], while the third reported lower alpha diversity, but statistical significance was not reported [55]. Similarly, two human studies did not show any difference [44,52], while one reported an increase in alpha diversity with B_{12} intake [45]. Regarding beta diversity, three studies did not show any difference [46,55,56], while one study reported different diversity at the genus level in mice [54]. In humans, two studies showed no difference [44,52], and one showed different beta diversity [45]. Therefore, while majority of the studies have reported similar findings as observed in the current study, others have observed differing outcomes, highlighting variability in the existing literature, possibly due to the difference in sequencing data, sample sizes, experimental variables.

The different 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 deficient group had a higher 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 [53]. 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 [57], 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, 58]. 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. We hypothesize that this association may reflect 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. An alternate hypothesis, which may however require more supporting evidence, can be a feedback response possibly originating from the host to the gut microbial community, in a way that enhances microbial B_{12} biosynthesis, so that it could source a fraction of the microbiota-derived B_{12} to meet its DRI and thereby partially prevent acute deficiency. If that being the case, then the gut microbiome would 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 remodeling 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) indicates a possibility of a threshold-dependent switch-like response (Supplemental Figure 4) 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 sparing raw material for *de-novo* Vit. B_{12} biosynthesis under severe micronutrient deficiency in the long term (Supplemental Figure 6).

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 [59]. 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 hypothesized 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

This study was based on case-control design, and hence cause-effect relationship can't be established. The results were based on a small sample size involving only children. The profiling of other biomarkers of Vit. B_{12} status (holotranscobalamin, methylmalonic acid, and homocysteine) and 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. To ascertain the proposed hypothesis and causality of the observed differences, longitudinal/interventional studies required to be done. Further, the

microbial signatures identified in our study could also 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 serum B_{12} level and gut microbiome at the species or strain level. The gut community in the B_{12}-deficient group was enriched with mucin-degrading bacteria, a few pathogens, and known B_{12}-producers. 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, which meets the community's demand for Vit. B_{12}.

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Authors' contributions

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.

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Table 1. List of the B_{12} 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**	

Table 2. Summary of the metadata collected from samples.

	Non-deficient (n=29)	**B_{12} deficient (n=30)**
Serum B_{12} level (pg/ml)	392.83 (±156.73)	161.63 (±30.80)
Age in years (mean± (sd))	6.4 (±2.6)	7.8 (±2.6)
Weight in kg (mean± (sd))	16.3 (±5.0)	18.8 (±4.8)
BMI (mean±(sd))	13.1 (±2.3)	13.9 (±2.1)
Gender	Female=16, Male=13	Female=21, Male=9
Diet	Nonvegetarian=24, Vegetarian=5	Nonvegetarian=30
Antibiotics	No	One subject took antibiotics 2 months before sampling

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 (*p-value = 0.05*), and **C** Shannon diversity (*p-value = 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 (*p-adj <= 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 (median contig coverage) 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. The y-axis is the median contig coverage.

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)*

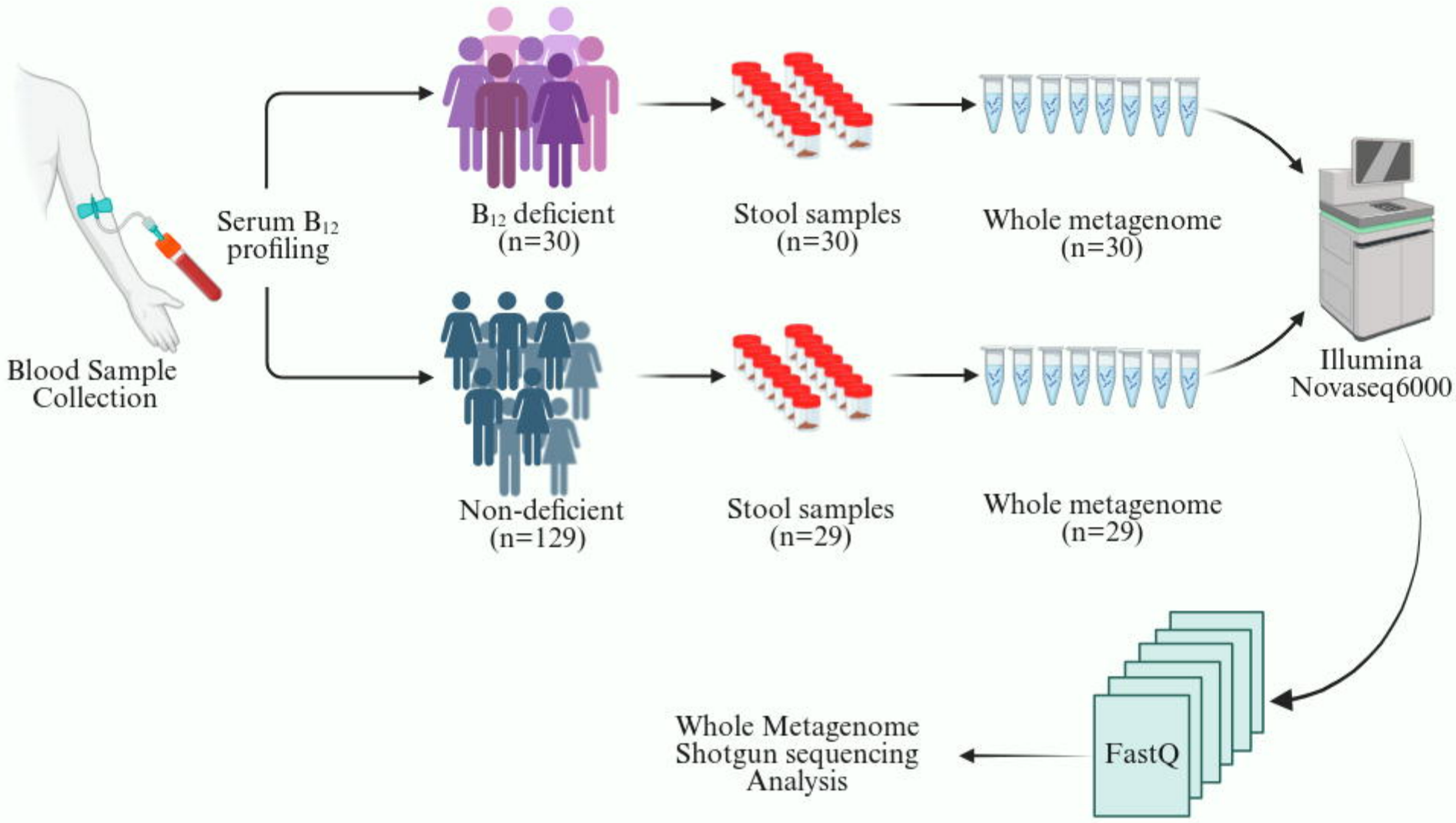
Supplemental Figure 4. Locally weighted scatterplot smoothing (LOWESS) of data in Figure

5B showed that the cumulative abundance of potential B_{12} producers (median contig coverage) increased non-linearly when the serum B_{12} level lies in the deficient range.

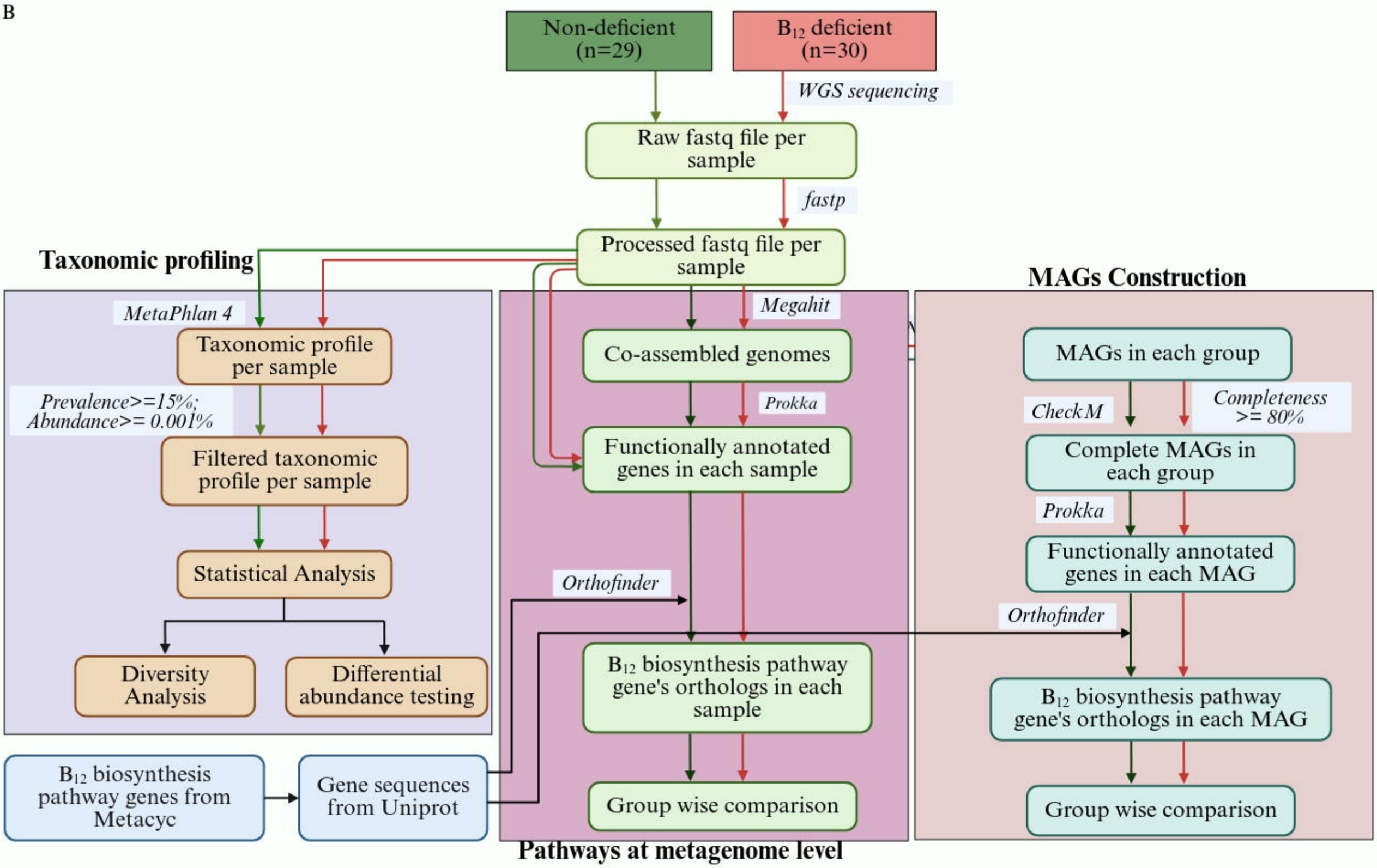
Supplemental Figure 5. Taxonomic classification of potential B_{12} producers at the phylum level in: A Non-deficient group, **B** B_{12} deficient group.

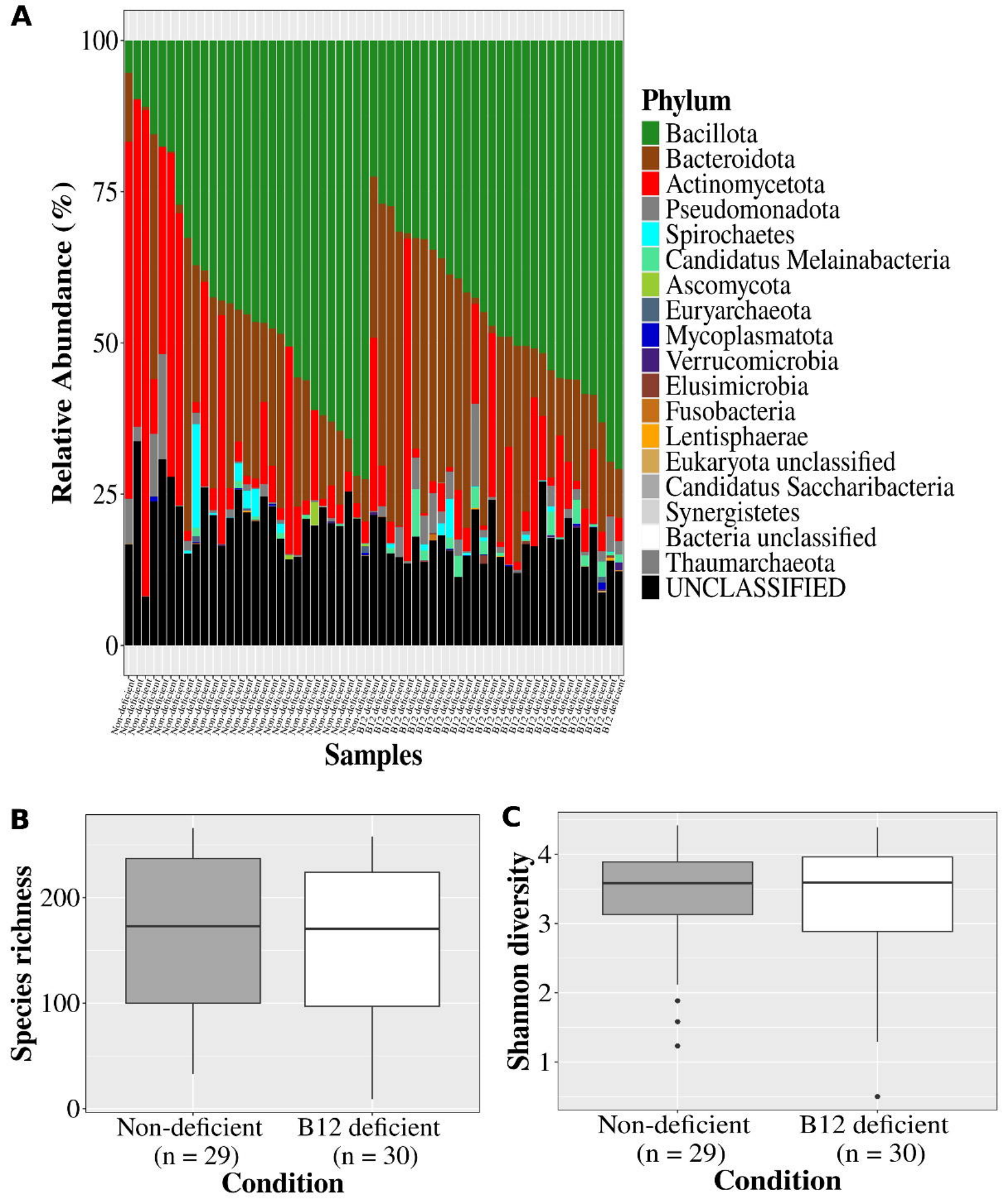
Supplemental Figure 6. The putative Host-microbiome feedback regulation loop **A.** In case of sufficient Vit. B_{12} bioavailability, the serum Vit. B_{12} is maintained above the deficiency threshold, wherein the microbiome shares the Vit. B_{12} from the host with a relatively lesser contribution 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 remodeling 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.

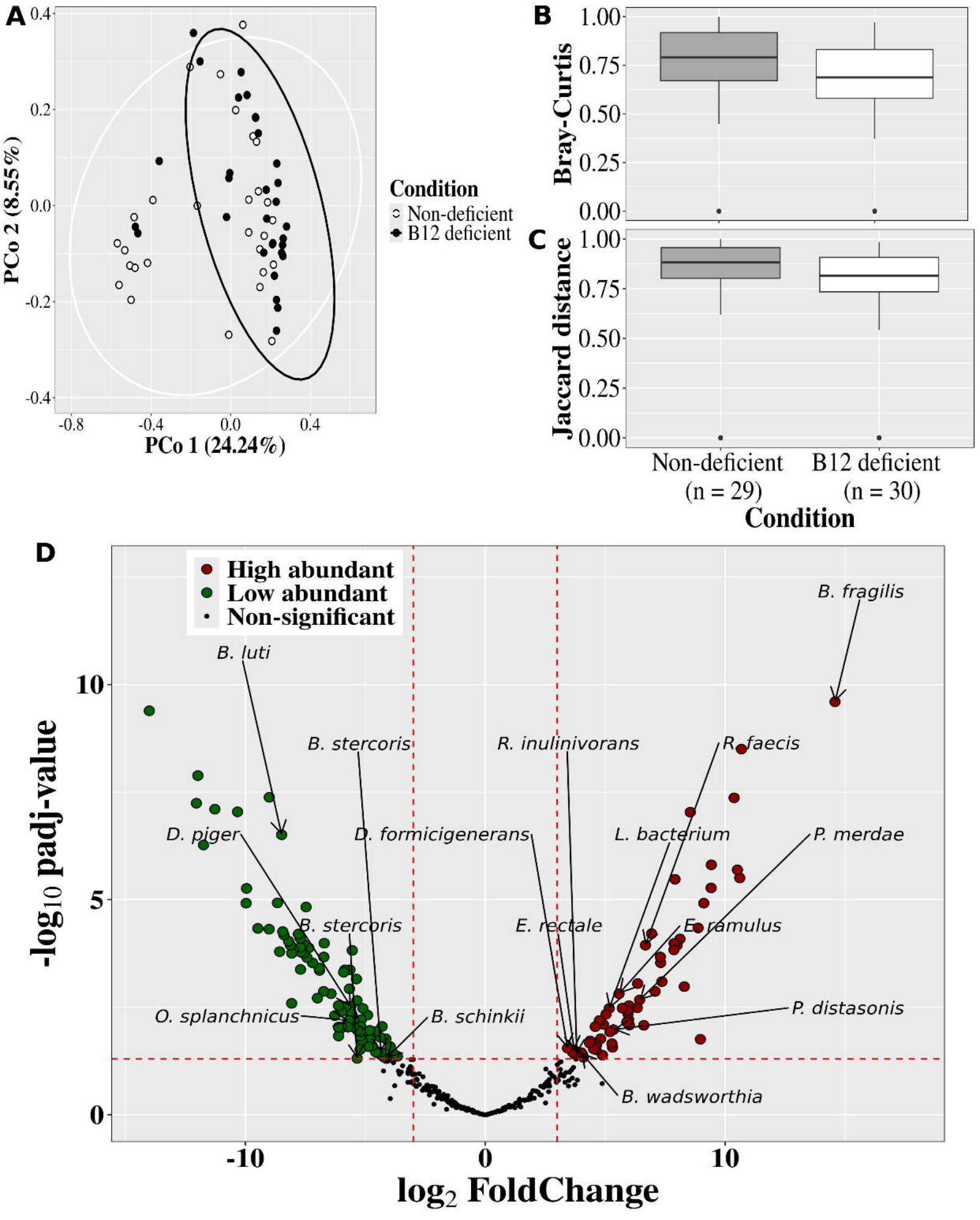
A

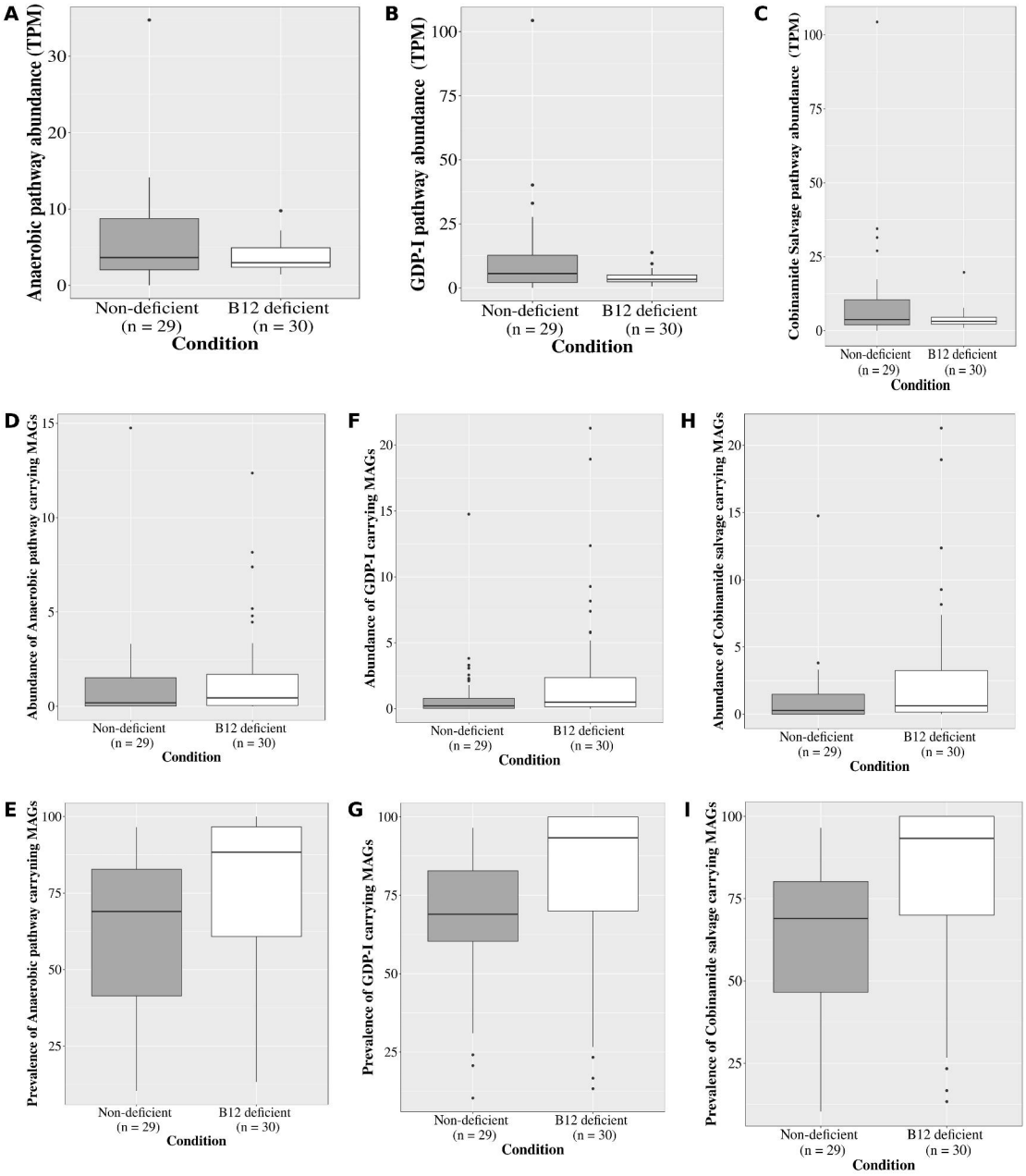

Blood Sample Collection
Serum B_{12} profiling
B_{12} deficient (n=30)
Non-deficient (n=129)
Stool samples (n=30)
Stool samples (n=29)
Whole metagenome (n=30)
Whole metagenome (n=29)
Illumina Novaseq6000
FastQ
Whole Metagenome Shotgun sequencing Analysis

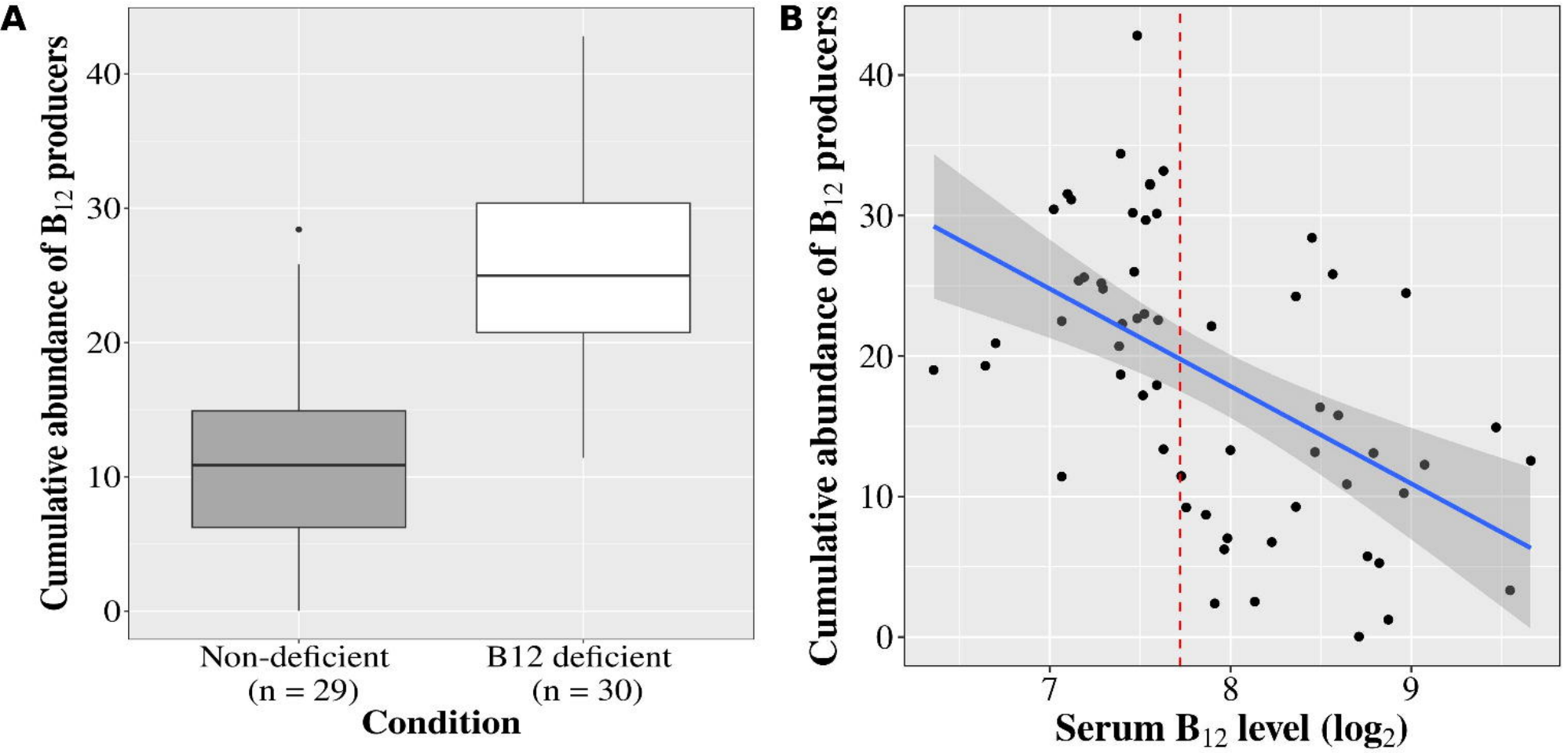
B


Non-deficient (n=29)
B_{12} 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
Co-assembled genomes
Prokka
Functionally annotated genes in each sample
Orthofinder
B_{12} biosynthesis pathway gene's orthologs in each sample
Group wise comparison
MAGs Construction
MAGs in each group
CheckM
Completeness >= 80%
Complete MAGs in each group
Prokka
Functionally annotated genes in each MAG
Orthofinder
B_{12} biosynthesis pathway gene's orthologs in each MAG
Group wise comparison
B_{12} biosynthesis pathway genes from Metacyc
Gene sequences from Uniprot
Pathways at metagenome level


A
Relative Abundance (%)
100
75
50
25
0
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
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
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
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
F
Abundance of GDP-I carrying MAGs
0
5
10
15
20
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
H
Abundance of Cobinamide salvage carrying MAGs
0
5
10
15
20
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
E
Prevalence of Anaerobic pathway carrying MAGs
25
50
75
100
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
G
Prevalence of GDP-I carrying MAGs
25
50
75
100
Non-deficient (n = 29)
B12 deficient (n = 30)
Condition
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
40
30
20
10
0
Non-deficient
(n = 29)
B12 deficient
(n = 30)
Condition
B
Cumulative abundance of B_{12} producers
40
30
20
10
0
7
8
9
Serum B_{12} level (log_2)