Title: Nutritional status impacts the gut microbiota of an adolescent refugee population.

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Abstract

Although the human gut microbiome, and its role in health and disease, have been extensively studied in different populations, a comprehensive assessment of gut microbiome composition has not been performed in vulnerable refugee populations. To address this shortfall, we explored gut microbiome diversity and associated demographic, health, and nutritional factors in adolescent Afghan refugees (n=206). Blood and faecal samples were collected and analysed for nutrition status markers and 16S rRNA gene amplicon-based community profiling, respectively. Bioinformatics and statistical analysis were performed using SPSS, QIIME and R. Overall, 56 distinct phyla, 117 families and 252 genera were identified in all faecal samples. Bacterial diversity (alpha and beta diversity) and *Firmicutes:Bacteroidetes* (F/B) were significantly higher in the 15–19 year old age group (cf. the 10-14 age group) but were lower in the underweight and vitamin D deficient groups. Furthermore, LEfSe analysis identified significant differences in the relative abundance of bacterial genera based on age, BMI, and micronutrients (vitamins and minerals) status. The results were further confirmed by correlation analysis wherein age, BMI and micronutrients status showed significant correlation (positive or negative) with F/B ratio and the relative abundance of specific bacterial taxa. Collectively, our study provides baseline gut microbiota profile and associated factors among adolescent Afghan refugees. These finding are crucial for translational microbiota research to improve the health of these understudied and vulnerable population.

Key words: Micronutrients deficiencies, Malnutrition, Afghan refugees, Zinc, Vitamin D, Selenium, Vitamin B12, Iron, Hemoglobin, Gut microbiome, Vulnerable

1. Background

The human gut contains trillions of microbial species (bacteria, viruses, fungi and archaea) that live in a mutually-beneficial, commensal relationship with the host and together constitute the gut microbiota (Qin et al., 2010). The human host offers the microbiota a warm and nutrient-rich environmental niche, while the gut microbiota provides essential nutrients (e.g. vitamins) and support host metabolism (Cox et al., 2022), immunity (Belkaid and Hand, 2014) and health (both physical and mental) (Cheung et al., 2019; Duvallet et al., 2017). The gut microbial ecosystem is relatively stable in healthy adults. However, changes in microbiota composition or its functional potential lead to microbial dysbiosis that has been implicated in a variety of human pathologies including inflammatory bowel disease, colorectal cancer, obesity, allergy and autoimmune diseases (Duvallet et al., 2017).

A growing body of research indicates that there are variations in gut microbiota diversity and composition between different human population groups (Gupta et al., 2017). Indeed, many factors contribute to variations in gut microbiota, and these can be broadly categorized into three types: geographical, ecological and host related. Until now, most research exploring gut microbiota diversity, function, and role in health and diseases has been focussed on host related factors such as genetics, ethnicity, diet, and lifestyle, but the role of geographic location combined with relocation (human migration) have received relatively little attention. Geographic location has been identified as an important factor affecting the diversity of the gut microbiome ecosystem (Cheng et al., 2022). A recent study (Lu et al., 2021) assessed the effect of 20 different variables including diet, lifestyle, ethnicity, and geographic location among 2678 healthy individuals belonging to eight ethnicities in 64 different cities in China. This study found that geographic location was one of the strongest drivers of microbiota diversity and this effect was linked to dietary and lifestyle factors (Lu et al., 2021).

The impact of geographic location on human microbiome diversity and function is well exemplified by cases of geographic relocation, such as cross-border movements, migration, refugeeism and enforced displacements. During the last five decades, the world has witnessed a three-fold increase in the number of migrants. According to the 2022 report of the United Nations High Commission for Refugees, around 108.4 million people were forcibly displaced across the globe (UNHCR, 2023). Of these, 35.3 million are refugees, i.e. individuals who fled their home countries due to war, violence, or well-founded fear of persecution. The life experiences of refugees are characterised by exclusion, xenophobia and global apathy (Tarnas et al., 2023). The majority live in refugee camps where they suffer from many problems including food insecurity, unhygienic conditions, poverty, psychological stress, pollution and limited access to healthcare provision (Gushulak et al., 2010). As a result, refugee populations are highly vulnerable to both communicable and non-communicable diseases, and the resultant detrimental health

consequences (WHO, 2022). Despite the importance of the gut microbiome in human health and disease, the status of the gut microbiome in these vulnerable population groups is rarely explored. There is thus a strong incentive to characterize the gut microbiome of apparently healthy but vulnerable refugee populations who take refuge in neighbouring, underdeveloped, and economically unstable countries, as in the case of Afghan refugees who have relocated to Pakistan.

Afghan refugees are the oldest and third largest refugee population in the world after those in Syria and Ukraine (UNHCR, 2023). Following the Soviet invasion of Afghanistan in 1978–79, millions of Afghanis fled to neighbouring countries, especially Pakistan, and settled there as refugees. The influx of Afghan refugees into Pakistan continued with the US/NATO/ISAF led invasion of Afghanistan in 2001. Currently, Pakistan hosts around 1.4 million registered and 1 million non-registered (illegal) Afghan refugees (Jamal, 2019). The refugee population in Pakistan constitutes a protracted, vulnerable community that faces numerous social and public health issues. We have recently reported the widespread prevalence of malnutrition and multiple micronutrient deficiencies in adolescent Afghan refugees within refugee camps in Peshawar, Pakistan (Saeedullah et al., 2021). However, until now, no study has evaluated the gut microbiome diversity of this population. This is surprising considering the central role that the gut microbiota plays in maintaining human health (Barone et al., 2022), particularly given the dependency of the gut microbiota on micronutrients and the high prevalence of micronutrient deficiencies in vulnerable refugee populations. In the current study, we have characterized gut microbiome diversity and its functional potential in adolescent Afghan refugees located in a refugee camp in Peshawar (Pakistan), and have provided stratification according to BMI, nutritional status, and micronutrient profile.

2. Methodology

2.1. Study population

This population-based, cross-sectional study was conducted in the Khazana refugee camp in District Peshawar, Pakistan from March to April 2020. As described previously (Saeedullah et al., 2021), a total of 206 participants (103 male and 103 female) fulfilling the following inclusion criteria were recruited: (a) apparently healthy adolescent children aged 10 – 19 years; (b) not taking any nutritional or micronutrient supplement; and (c) living in the study area for at least one year. Non-consenting participants and those who were not capable of giving informed consent, were excluded, as were those taking antibiotics, prebiotics, or probiotics (in food products or as supplements), laxatives, antispasmodics, or anti-diarrhoea drugs (e.g. Orlistat or

Lactulose) either during the study period or in the previous two months. The study followed the ethical guidelines outlined in the Helsinki Declaration and ethical approval was granted by the Ethics Board of Khyber Medical University, Peshawar (DIR/KMU-EB/PR/000766). Before the data and sample collection, written informed consent was obtained from either the participant, parents, or legal guardians, depending on the age of the participant.

2.2. Demographic information and anthropometric measurement

Socio-demographic information of each participant was collected by trained research assistants using an interviewer-administered, paper-based questionnaire. Anthropometric measurements, including height and weight, were recorded following standard methods. For measuring height, the participants were asked to remove shoes, head scarves, caps etc, and stand against a wall-mounted height stadiometer in the Frankfurt position. The measuring scale was lowered until it rested on the scalp and measurements were recorded to the nearest 0.1 cm. For recording weight, the participants were instructed to remove extra clothing, jewellery, and shoes and stand on an electronic scale (Secca, UK). The weight was recorded to the nearest 0.1 1 kg. For consistency and accuracy, both height and weight were recorded three times, and the average was considered for final calculations. Body mass index (BMI) was calculated by dividing the weight in kg by the squared height in meters (kg/m^2).

2.3. Collection of biological samples

Blood and faecal samples were collected from all the participants. Non-fasting, whole-blood samples (2 x 5 mL) were collected by a trained phlebotomist in the morning between 7 – 10 am using a butterfly needle, vacutainer and two pre-chilled tubes, one containing EDTA and one with silica-clot activator. The samples were kept on ice during collection, centrifuged at 3000 rpm for 10 minutes, aliquoted into 0.5 mL portions in 1 mL cryotubes and then frozen at -80 °C. For collection of faecal samples, each participant was provided with a stool sample collection kit including a collection pot, disposable gloves, a screw-top tube, and a detailed instructions sheet describing how to collect the stool sample. The participants were instructed to wear gloves and pass an entire bowel movement into the stool collection pot. Once the stool sample was collected, the participants were required to: open the screw-top tube (10 mL); fill at least half of the tube using the attached mini-spoon; secure the lid tightly and then place the tube in the plastic bag provided; and finally, handover the tube to the researcher within one hour of sample collection. The participants were instructed to wash their hands with soap after collecting the stool sample. To preserve the DNA integrity, the samples were mixed with an equal volume of DNA/RNA shield (Zymo Research, USA), transported to the main laboratory, and stored in the refrigerator until further analysis.

2.4. Laboratory analysis

Haematological parameters, including complete blood count, haemoglobin, haematocrit and mean corpuscular volume, were assessed using freshly collected whole-blood samples using an automated haematology analyser (Sysmex XP-100, 19 Jalan Tukang, Singapore). A 25-OH vitamin D Diasorin radioimmunoassay ELISA kit (Euroimmun, Germany) was used to assess plasma vitamin D status following the manufacturer's instructions. Ferritin, vitamin B12 and folate levels were assessed in serum using the Abbott Architect i2000 analyzer (Abbott Diagnostics, Zug, Switzerland).

Elemental concentrations in plasma samples were determined using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Bremen, Germany). Freeze-dried samples were reconstituted using Milli-Q (18 MΩ) water prior to analysis. The samples were introduced, via a single line, from an autosampler incorporating an ASXpress™ rapid uptake module (Cetac ASX-520, Teledyne Technologies Inc., Omaha, NE, USA) through a perfluoroalkoxy (PFA) Microflow PFA-ST nebulizer (Thermo Fisher Scientific, Bremen, Germany). All samples and external multi-element calibration standards were diluted in a solution containing (i) 0.5% HNO_3 (*Primar Plus* grade), (ii) 2.0% methanol (Fisher Scientific UK Ltd, Loughborough, UK) and (iii) three internal standards (^{72}Ge (10 µg L^{-1}), ^{103}Rh (5 µg L^{-1}), ^{193}Ir (5 µg L^{-1})) (SPEX Certiprep Inc., Metuchen, NJ, USA). Calibration standards included (i) a multi-element solution with Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl, U, V and Zn, in the range 0 – 100 µg L^{-1} (0, 20, 40, 100 µg L^{-1}) (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA); (ii) a bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, France) with Ca, Mg, Na and K in the range 0-30 mg L^{-1} and (iii) a mixed phosphorus, boron and Sulphur standard made in-house from salt solutions (KH_2PO_4, K_2SO_4 and H_3BO_3). The ICP-MS was operated in 'collision-reaction cell mode', with kinetic energy discrimination, using H_2 as the cell gas to maximize sensitivity for Se determination and He for all other elements. Accuracy was verified using two reference materials (Seronorm™ L-1 (Lot 1801802) and Seronorm™ L-2 (Lot 1801803)); Nycomed Pharma AS, Billingstad, Norway); these were run at the start and the end of sample batch runs and were prepared identically to reconstituted samples and calibration standards. A total of 8 individual CRM analyses were undertaken for both L-1 and L-2. The average recoveries recorded for Seronorm™ L-1 and Seronorm™ L-2 are given in Table 1. This procedure was designed to ensure accurate and precise elemental analysis, providing reliable results for the specified elements.

Table 1: Average recovery (%; n = 8) for eight elements compared to accredited values determined across 3 analytical batches of blood plasma.

CRM % Recovery	Mg	Ca	Mn	Fe	Co	Cu	Zn	Se
Seronorm L-1	95	91	100	93	102	88	91	101
Seronorm L-2	101	89	102	95	106	88	90	104

2.5. DNA extraction and 16S rRNA gene amplicon sequencing

Total DNA was extracted using a QIAmp Fast DNA Stool Mini Kit following the manufacturer's instructions, with slight modifications. Samples preserved with DNA shield in 1.5 mL Eppendorf tubes were defrosted on ice and centrifuged at 13000 g for 1 min. The pellet was then washed with 0.5 mL PBS and resuspended in 1 mL of InhibitEX buffer, and the suspensions were transferred to new tubes containing acid washed ≤ 100 µm glass beads (Sigma). The samples were placed into an adapter (24 X 2 mL tube holder) of a FastPrep-24 5G bead beater (MP Biomedicals) and beating was applied at a speed of 6 m/s for 60 s. Samples were then maintained on ice for a further 60 s. This step was repeated three times before samples were vortexed and centrifuged at 13000 g for 1 min. The supernatant (0.6 mL) was transferred to fresh tubes with 25 µL of Proteinase K reagent. This was followed by the addition of 0.6 mL AL buffer before vortexing for 15 s. Samples were incubated in a water bath (70 °C) for 10 min and 0.6 mL of ethanol (96-100%) were added to the lysate before vortexing. Carefully, 0.6 mL of the lysate were transferred to the QIAamp spin column before being centrifuged at 13000 g for 1 min. The tubes containing the filtrate were discarded and the spin columns transferred to fresh 2 mL Eppendorf tubes. This step was repeated until all the lysate has been fully loaded into the spin column. Then, 0.5 mL of Buffer AW1 were added to the spin column and this was centrifuged at 13000 g for 1 min, after which the filtrate was discarded. The spin columns were then placed in fresh 2 mL collection tubes and 0.5 mL of AW2 buffer added. The tubes were centrifuged for 3 min at 13000 g and the collection tubes containing the filtrate discarded. The spin columns were placed in fresh 2 mL collection tubes and centrifuged for 3 min at 13000 g. The QIAamp spin columns were transferred to fresh 1.5 microcentrifuge tubes and 0.03 mL of distilled water were added to the columns, which were then incubated at room temperature for 1 min followed by centrifugation at 13000 g for 1 min. After centrifuging, the previous step was repeated with 0.03 mL of distilled added to the spin column, incubated at room temperature for 1 min and then centrifuged at 13000 g for 1 min. The spin column was discarded and the 0.06 mL of collected filtrate, in the 1.5 microcentrifuge tube, was then stored at -20 °C until submission on dry ice for NGS. DNA quality was assessed by agarose gel electrophoresis, Nanodrop and PCR amplification. 16S rRNA gene amplicon-based sequencing was performed by Animal and Plant Health Agency (APHA, UK) using an Illumina MiSeq platform to generate paired-end reads. The amplicon libraries were created

from the V3-V4 hypervariable regions of the bacterial 16S rRNA gene using 349F (GYGCASCAGKCGMGAAW) and 786R (GGACTACVSGGGTATCTAAT) primers before 16S rRNA sequencing.

2.6. Statistical analysis

2.6.1. Descriptive statistics

Demographic characteristics of the study participants were summarized using descriptive statistics (mean, standard deviation, and frequencies). Chi-square and the Student's t-test were used to determine the differences between continuous and categorical variables, respectively, and a p value of <0.05 was considered significant.

2.6.2. Bioinformatics data analysis

The 16S rRNA gene sequencing data were analyzed with DADA2 (Callahan et al., 2016) software to determine sequencing error rates, dereplicate amplicons and remove chimeric sequences. The reads were trimmed to 280 and 160 bp for forward and reverse reads, respectively, to obtain high-quality sequences (phred >30). In addition, the reads with N nucleotides and >2 expected errors were discarded (maxN = 0, maxEE = 2, truncQ = 2) and the final clean reads data are presented in Supplementary table 1. The resulting clean and high-quality reads were used to assign taxonomy using the k-mer based tool Kraken2 (v2.1.2), followed by relative abundance estimation with Bracken (v2.8) (Lu and Salzberg, 2020) at phylum, class, order, family and genus level specifying the minimum number of reads required for classification at the specified rank (threshold = 5). Taxonomic classification and abundance estimation were performed using the Greengenes Database (v13.5) for Kraken2 (Lu & Salzberg, 2020). Read count data obtained from the Kraken2 analysis was used for alpha diversity measures and differences in the alpha diversity index between groups were analyzed by Wilcox test (significance <0.05). Relative abundance data obtained from Bracken were used to generate multidimensional scaling (MDS) plots for the comparison of β diversities based on Bray–Curtis dissimilarity matrices using the vegan package (v2.6.4). Linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed with Phyloseq (v1.40.0). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2 v2.5.2) software was employed for predicting the functional abundances based on marker gene sequences (Douglas et al., 2020). PICRUSt2 results were visualized with ggpicrust2 (v1.7.2) vignette (Yang et al., 2023). All analyses were implemented in R version 4.2.3.

3. Results

3.1 Population characteristics

From the total number of 206 participants, those with low-quality stool-sample DNA sequence data (n=10) were excluded from all further analyses. The sociodemographic characteristics and

plasma micronutrient data for the remaining study participants (n=196) are presented in Table 2. The mean age of the study participants was 13.4 ± 2.9 years with no significant differences between female and female participants. The majority (n=126; 64.3%) of the participants were in the age range 10 - 14 years, living in a large family size with more than 10 family members (n=101; 51.8%) and were educated to primary level (n=118; 60.5%). Socioeconomically, the sample population was homogenous with almost three out of four participants having a monthly household income of <25000 Pakistani rupees (the minimum wage). Half of the study population (n = 101; 51.8%) were underweight. Multiple micronutrients deficiencies were also common.

Table 2: Demographic characteristics of the study participants

Characteristics		Total n (%)	Gender		P-value*
			Male n (%)	Female n (%)	
Age categories	10 - 14	126 (64.3)	67 (71.3)	59 (57.8)	0.05
	15 - 19	70 (35.7)	27 (28.7)	43 (42.2)	
Family size	1 - 4	6 (3.1)	5 (5.4)	1 (1)	NS#
	5 - 9	69 (35.4)	35 (37.6)	34 (33.3)	
	10 - 19	101 (51.8)	46 (49.5)	55 (53.9)	
	20 & above	19 (9.7)	7 (7.5)	12 (11.8)	
Education Categories	No formal education	39 (20)	8 (8.6)	31 (15.9)	0.001
	Primary level	118 (60.5)	62 (66.7)	56 (54.9)	
	High school level	36 (18.5)	21 (22.6)	15 (14.7)	
	College & university	2 (1)	2 (2.2)	0 (0)	
Income categories	<25000 PKR	143 (73.3)	66 (71)	77 (75.5)	NS
	≥ 25000 PKR	52 (26.7)	27 (29)	25 (24.5)	
Main source of drinking water	Hand pump	191 (97.4)	92 (97.9)	99 (97.1)	NS
	Covered well	3 (1.5)	1 (1.1)	2 (2)	
	Motor pump	1 (0.5)	1 (1.1)	0 (0)	
BMI categories based on Asian cut offs	Normal weight	79 (40.5)	32 (34.4)	47 (46.1)	NS
	Underweight	101 (51.8)	54 (58.1)	47 (46.1)	
	Overweight	13 (6.7)	5 (5.4)	8 (7.8)	
	Obese	2 (1)	2 (2.2)	0 (0)	
Anemia	Yes	19 (10.5)	10 (12.7)	9 (8.8)	NS
	No	162 (89.5)	69 (87.3)	93 (91.2)	
Presence of Vit-D deficiency	Yes	149 (82.3)	64 (73.6)	85 (90.4)	0.003
	No	32 (17.7)	23 (26.4)	9 (9.6)	
Depleted iron stores based on serum ferritin concentration (<15 ng/mL)	Yes	18 (9.6)	4 (4.4)	14 (14.4)	0.025
	No	170 (90.4)	87 (95.6)	83 (85.6)	
Vitamin b12 deficiency based on WHO criteria of <203pg/mL)	No	104 (57.1)	39 (44.8)	65 (68.4)	0.002
	Yes	78 (42.9)	48 (55.2)	30 (31.6)	
Folate level	Normal (6 - 20 ng/mL)	53 (27)	15 (16)	38 (19.4)	0.002
	Possible Deficiency (3 - 5.9 ng/mL)	114 (58.2)	56 (59.6)	58 (56.9)	
	Deficiency (<3 ng/mL)	29 (14.8)	23 (24.5)	6 (5.9)	
Copper level	Normal	141 (73.8)	68 (74.7)	73 (73)	NS
	Deficiency	50 (26.2)	23 (25.3)	27 (27)	
Zinc level	Normal	123 (64.4)	62 (68.1)	61 (61)	NS
	Deficiency	68 (35.6)	29 (31.9)	39 (39)	
Selenium deficiency	Normal	94 (49.2)	56 (61.5)	38 (38)	0.001
	Selenium	97 (50.8)	35 (38.5)	62 (62)	
Manganese categories	High	67 (34.2)	40 (42.6)	27 (26.5)	NS
	Low	4 (2)	1 (1.1)	3 (2.9)	
	Normal	120 (61.2)	50 (53.2)	70 (68.6)	
Chromium categories	Normal	6 (3.1)	4 (4.3)	2 (2)	NS
	Low	180 (91.8)	85 (90.4)	95 (93.1)	
	High	5 (2.6)	2 (2.1)	3 (2.9)	
	Missing	5 (2.6)	3 (3.3)	2 (2)	

*The Chi-square test was used to calculate differences between the male and female groups. #NS; non-significant

3.2 Gut microbiome composition and associated covariates

Of the total 206 faecal samples subjected to sequencing analysis, 196 samples (102 female and 94 male) yielded good quality sequencing reads and were therefore included in the final analysis. In total, 8.7 million quality sequence reads were obtained with an average of 44000 reads per sample (range 14726 – 123047). The overall reads statistics are presented in Supplementary Table 1. The gut microbiome samples of adolescent Afghan refugees displayed diverse bacterial flora at all taxonomic levels**.** Overall, a total of 56 distinct phyla, 117 families and 252 genera were identified in all faecal samples. Overall, *Firmicutes* (82%)*, Bacteriodetes* (13.2%) and *Actinobacteria* (2.9%) were the most abundant bacterial phyla contributing to 98% of the reads (Fig 1A). On genus level, *Faecalibacterium* (20%), *Prevotella* (12.5%), *Blautia* (11.8%), *Catenibacterium* (9.0%) and *Ruminococcus* (7.5%) were the five most abundant bacterial genera representing 60% of the gut microbiome communities in the Afghan refugee population (Fig 1C).

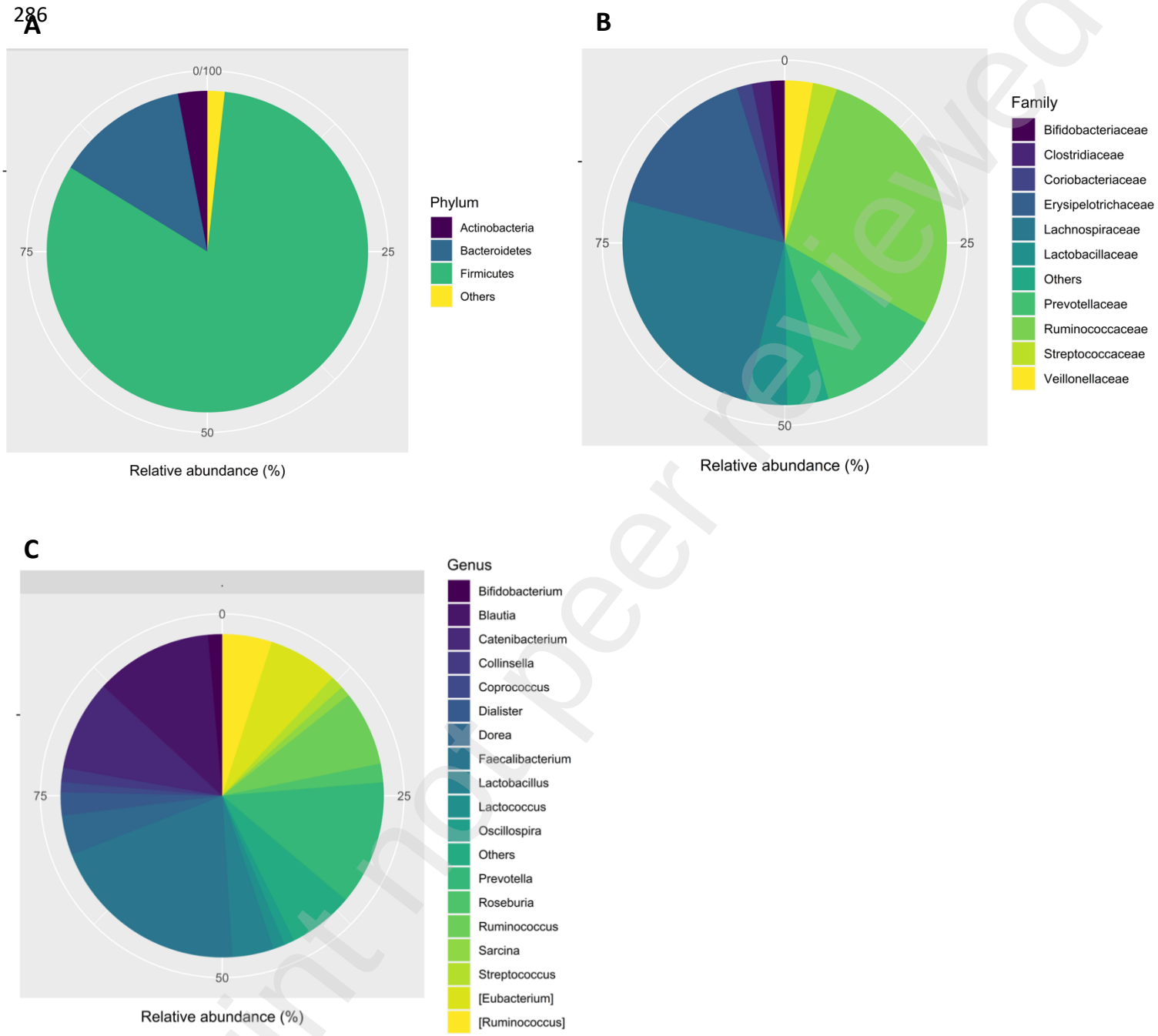

Figure 1: Taxonomic annotation and abundance of the overall data from the study participants. The Pie Charts showing the microbial taxa profile in the dataset at the (A) phylum and (B) Family and (C) genus levels. “Others” indicates rare taxa (< 1% relative abundance).

3.3 Impact of age and gender on gut microbiota

We next assessed age and gender-based differences in the gut microbiome diversity and functions in the sample population. As shown in Figure 2A, the alpha diversity measures, assessing species richness and evenness (Observed, Chao1, Shannon and Simpson), were higher in the 15 – 19 age group compared to 10 – 14 age group with significant differences in Observed diversity. Moreover, the beta diversity index, which is a measure of dissimilarity in community composition, also showed significant differences ($p = 0.006$) based on multidimensional scaling (MD S) of beta diversity analysis (Fig 2B). To further differentiate taxonomic differences between the gut microbiota of the two age groups, we used linear discriminant analysis (LDA) effect size (LEfSe). LDA score indicates the effect size of each of the abundant genera. Fig 2C presents the difference in abundance between the two age groups at the genus level with corresponding LDA scores. The gut microbiomes of the 10-14 age group showed significant enrichment of 11 bacterial genera with *Prevotella_9, Prevotella* and *Dialester* being the top three most abundant genera. In the 15 – 19 age group, *Bifidobacterium*, *Dorea* and *Romboutsia* were the top three enriched genera. The *Firmicutes*:*Bacteroidetes* ratio (F/B ratio), the most widely used indicator of gut microbial dysbiosis (Stojanov et al., 2020), was significantly higher in the 15 – 19 age group compared to 10-14 age group (Fig 2D).

The male and female groups showed no overall significant gender-based differences in gut microbiota alpha or beta diversity or F/B ratio. However, the gut microbiota of the female participants showed significant enrichment of 15 bacterial genera (LDA score >2, $P<0.05$). Of these, Enterococcus*, Escherichia-Shigella, Bacteroides, Terrsiporobacter* and *Intestinibacter* were the five most abundant genera (Supplementary Figure S1A-D).

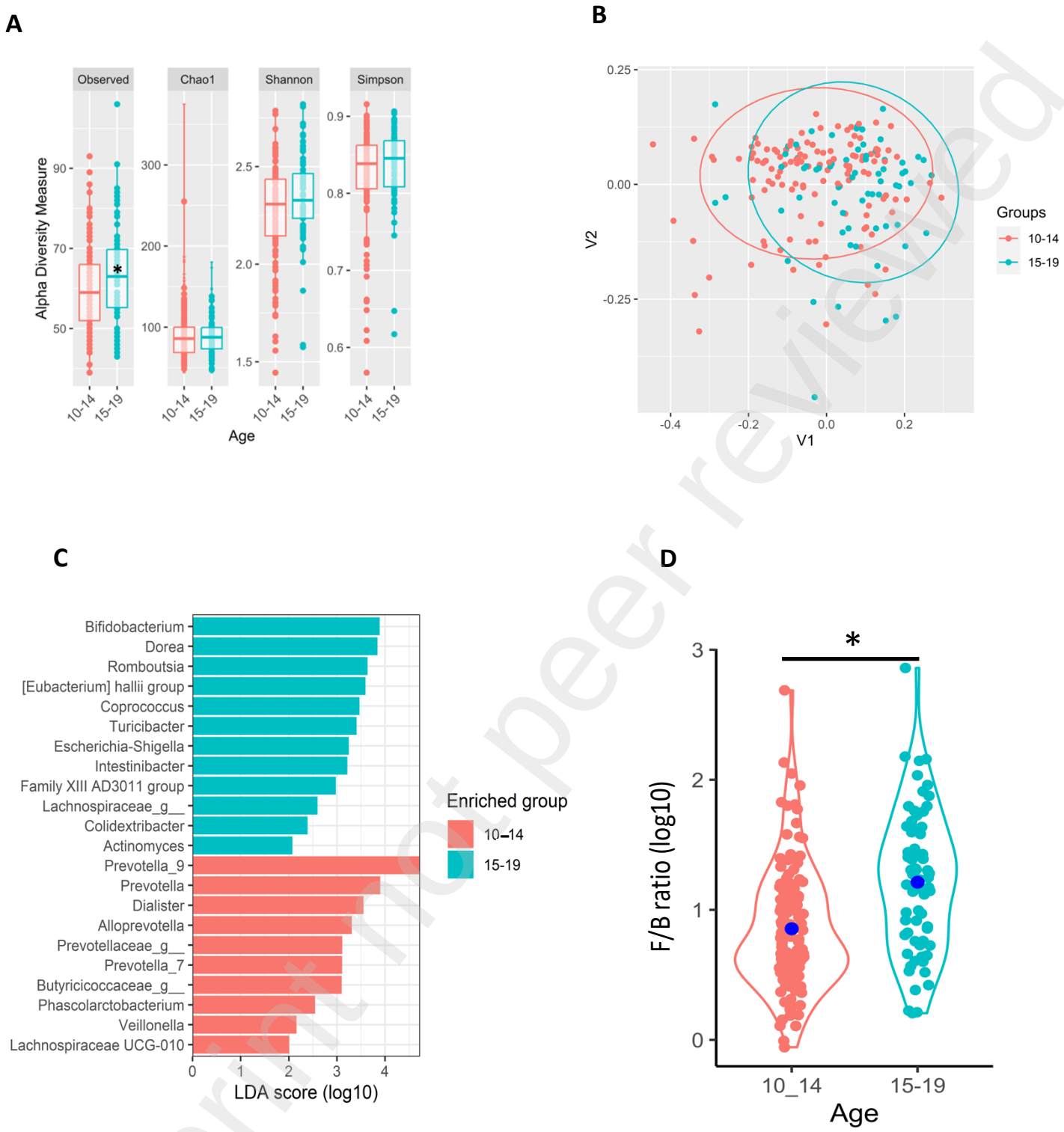

Figure 2: Taxonomic diversity, composition and abundance profiling of the study participants based on age groups. **(A)** Alpha diversity measures (* show statistical significance between age groups at $p < 0.05$). **(B)** Bray-Curtis β diversity between the age groups. **(C)** Linear discriminant analysis (LDA) distribution of LEfSe results based on the classification information at genus level. The threshold value of the LDA score was set to 2 and an LDA score >2 was considered significant. **(D)** Violin plot showing Firmicutes/Bacteroidetes ratio between the two age groups (* shows $p<0.05$).

3.4 Association between BMI and gut microbiota

The study participants were divided into underweight and normal BMI groups (n=79 and n=101 respectively) using the WHO cut-off values. Overweight and obese participants were excluded from the analysis due to low numbers (n=15). Overall, genus richness and diversity, as assessed by Observed, Chao1, Shannon and Simpson indices, were higher in the normal weight than the underweight group. However, the differences in diversity indices between the two groups were only significant for the Shannon index (Fig 3A). The NMD beta diversity analysis showed no apparent stratification of gut microbiome composition based on BMI (Fig 3B). Compared to the underweight group, the gut microbiome of normal weight adolescents was highly enriched (LDA score >2; $p<0.05$) with bacteria belonging to the *Lactobacillus, Megasphaera, Butyrivibrio, Roseburia* and *Peptococcus* genera and depleted in *Odoribacter* spp. (Fig 3C). The F/B ratio was also slightly higher in the normal weight individuals, although this difference was not significant (Fig 3D).

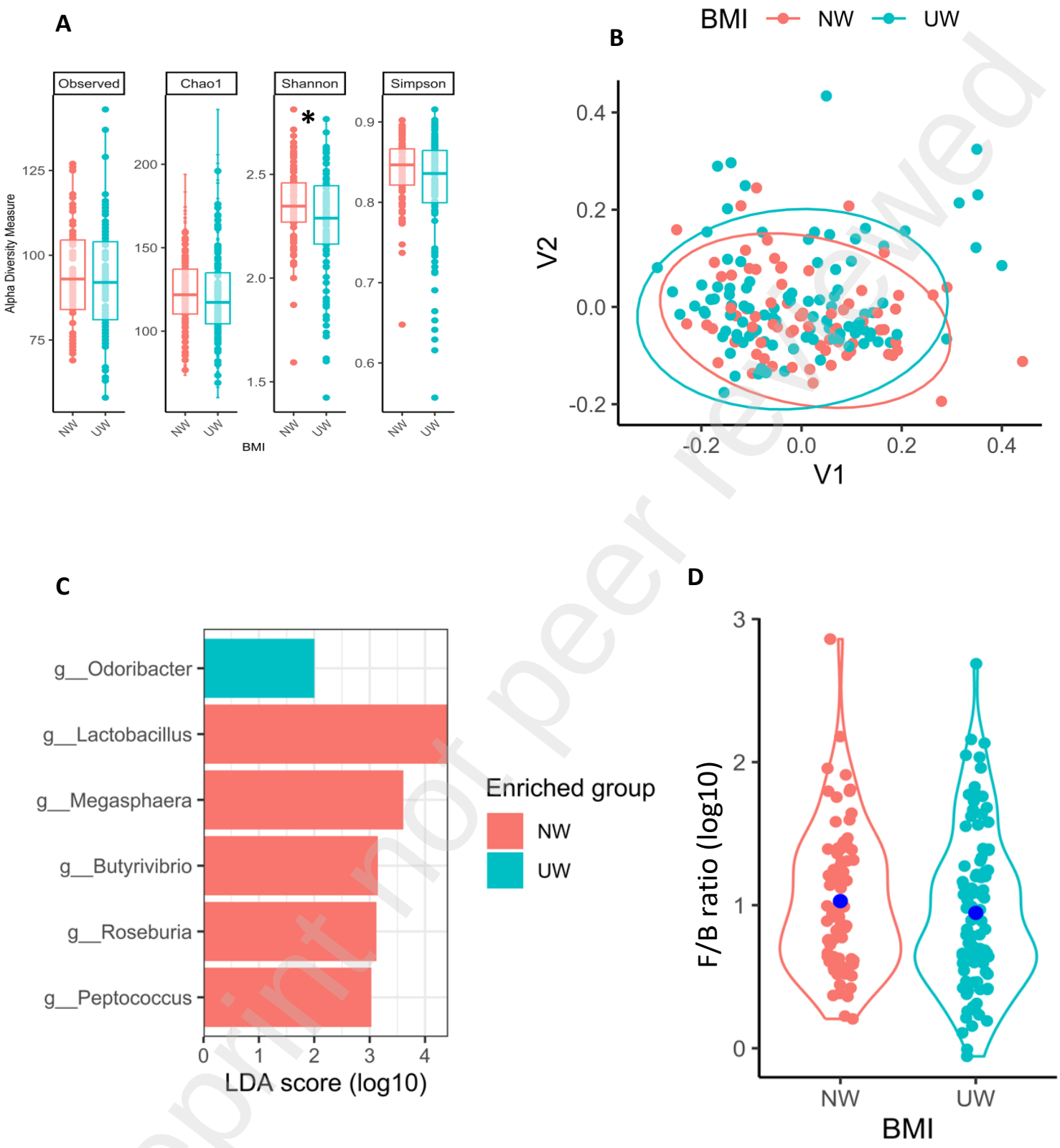

Figure 3: Bacterial diversity, composition, and abundance profile of study participants on the basis of BMI. (A) Alpha diversity measures (*, significant difference $p<0.05$). **(B)** Bray-Curtis β diversity between the normal weight and underweight adolescents. **(C)** Linear discriminant analysis (LDA) distribution of LEfSe results based on classification at genus level. An LDA score >2 was considered significant. **(D)** Differences in the Firmicutes/Bacteroidetes ratio between the two BMI groups.

3.5 Association between iron status and the gut microbiome

Serum levels for haemoglobin and ferritin were used as biomarkers of iron status. Based on the age- and gender-based WHO standard cut offs, around 10% of the study participants were found to be anaemic and possess a low serum ferritin level suggestive of deficient body iron stores (Table 1). Observed richness, diversity and composition were not significantly different between anaemic and non-anaemic participants (Fig 4A-B). However, LEfSe analysis revealed that for the gut microbiota of anaemic individuals, there was an enrichment of 12 and depletion of four bacterial genera (Figure 4C). Similar, nonsignificant differences in alpha and beta diversity were also observed between the participants with normal and low serum ferritin levels (Fig 4DE). In addition, the gut microbiome of the low serum ferritin individuals showed significantly lower abundance (LDA score >2; $p<0.05$) of six genera (*Blautia*, *Dorea*, *Butyricicoccus, Sarcins, Fusicatenibacter* and *Klebsiella*) along with enrichment of the *Alloprevotella*, *Catenisphaera* and *Oribacterium* genera (Fig 4F)

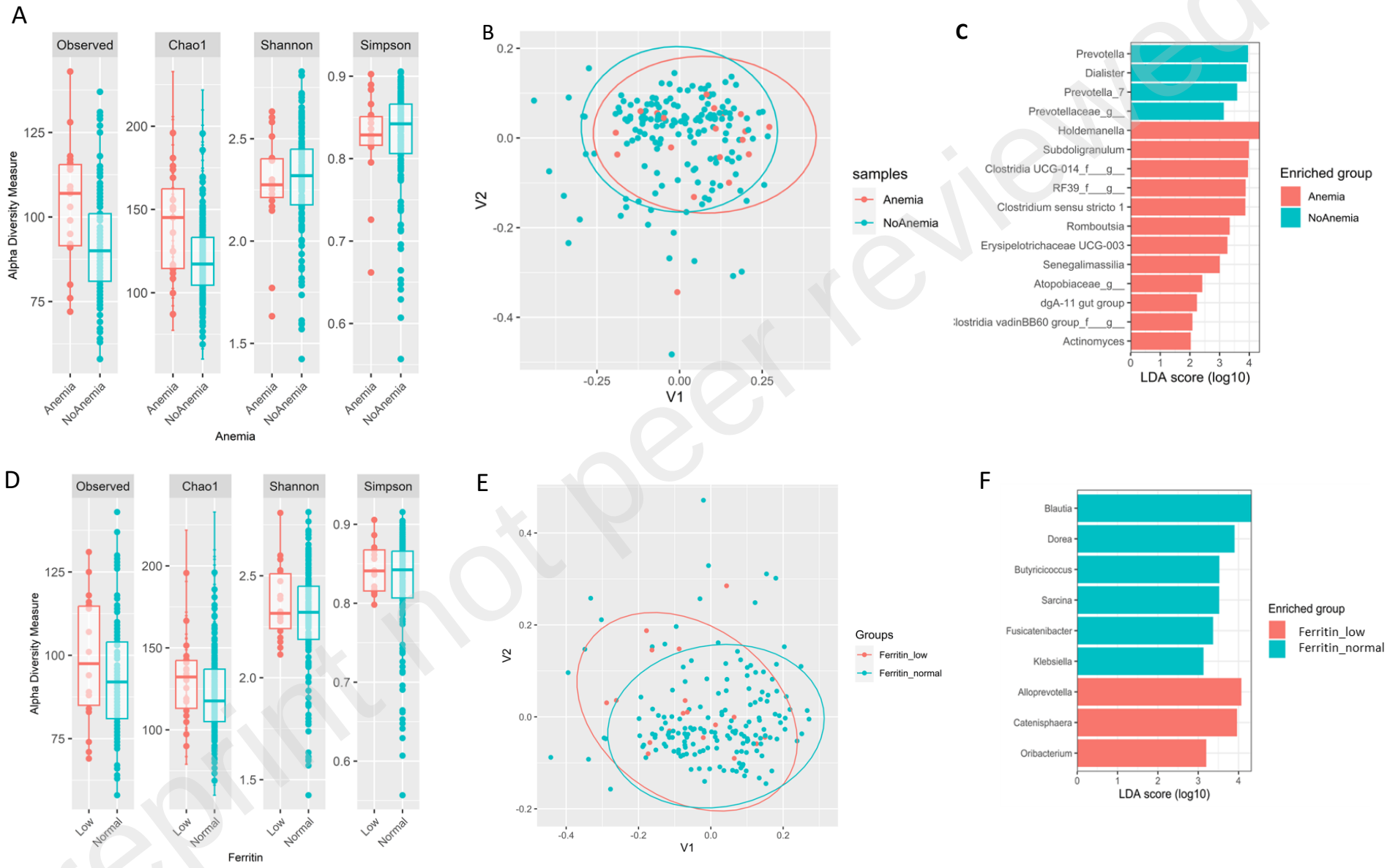

Figure 4: Bacterial diversity, composition, and abundance profile of study participants based on anemia and serum ferritin. **(A)** Alpha diversity measures based on anemia. **(B)** Bray-Curtis β diversity between participants with and without anemia. **(C)** Linear discriminant analysis (LDA) distribution of LEfSe results based on the classification information at the genus level for the non-anemic and anemic groups. An LDA score >2 was considered significant. **(D)** Alpha diversity measures based on normal or low serum ferritin levels. **(E)** Bray-Curtis β-diversity between participants with normal and low serum ferritin. **(F)** Linear discriminant analysis (LDA) distribution of LEfSe results based on the classification information at the genus level for the normal and low serum ferritin groups.

3.6 Impact of vitamin levels on gut microbiome

Next, we assessed the relationship/association between gut microbiota composition and serum vitamin D, vitamin B-9 and vitamin B-12 levels in the adolescent Afghan refugees. As shown in Fig 5 A- D, vitamin D status showed an impact on gut microbiota as indicated by a significantly lower Chao1 diversity index (Fig 5A) and enrichment of *Peptococcus* and *Methanobrevibacter* genera, and depletion of the *Weissella* genus (Figure 5C) in vitamin D deficient individuals compared to the sufficient group. Serum vitamin D levels had no significant impact on beta diversity and F/B ratio (Fig 5B & D). Similarly, gut microbiota diversity indices (Alpha and Beta) and F/B ratio were not significantly different between the normal and vitamin B12 (cobalamin) and vitamin B-9 deficient individuals (Supplementary Fig S2 and S3). However, significant differences were observed in the relative abundance. There was an enrichment of the *Blautia, RF-39, Anaerostipes* and *Gastranaerophilales* genera and depletion of *Escherichia-Shigella, Turicibacter* and *Howardella* in the vitamin B12 deficient group (Supplementary Fig S2). The *Shuttleworthia, Phasecolarctobacterium* and *Bulleidia* genera were highly enriched in the folate deficient group (Supplementary Fig S3).

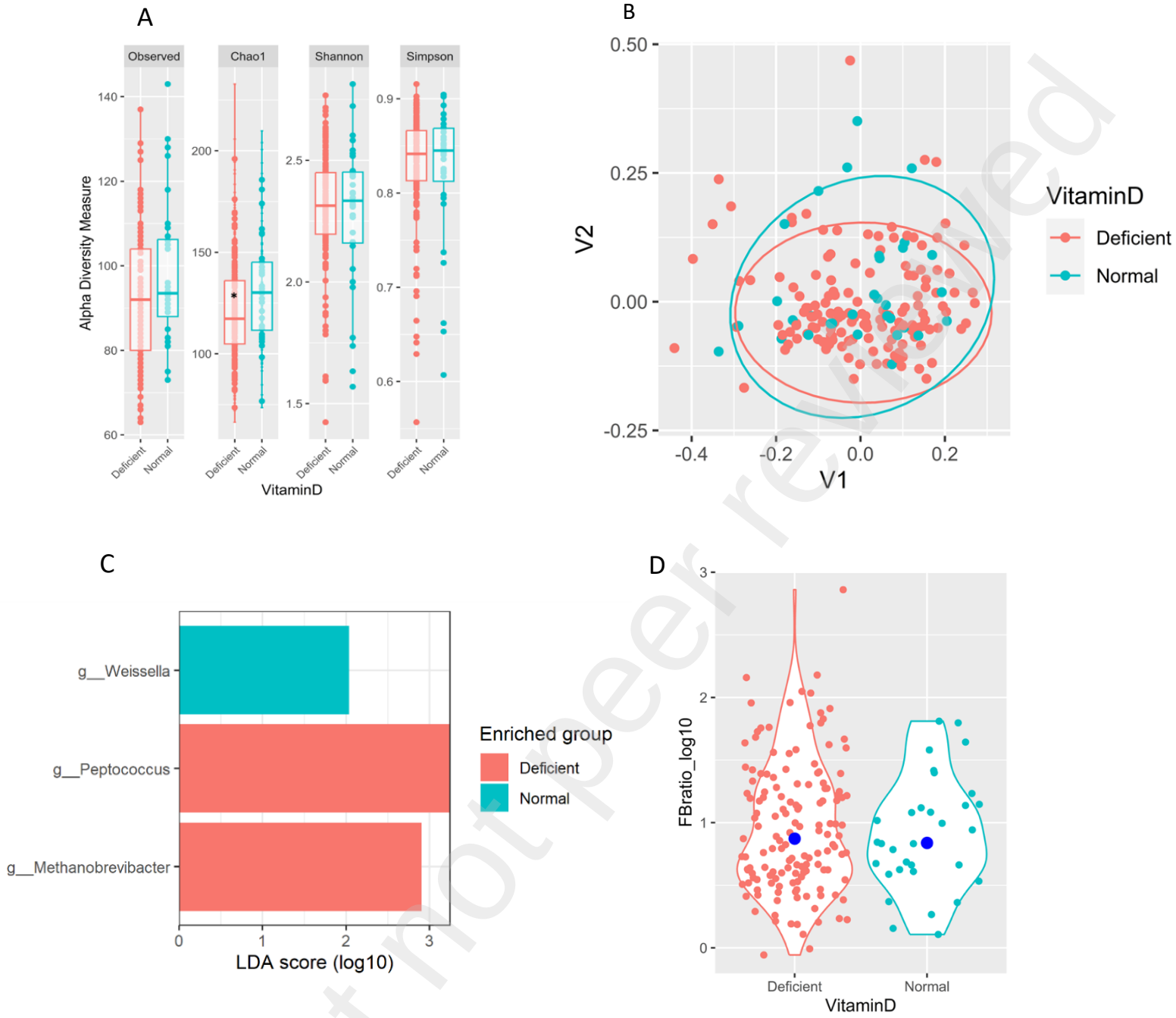

Figure 5: Bacterial diversity, composition, and abundance profile of study participants based on vitamin D deficiency. (A) Alpha diversity measures based on vitamin D. **(B)** Bray-Curtis β diversity between normal and vitamin D deficient individual. **(C)** Linear discriminant analysis (LDA) distribution of LEfSe results based on the classification information at the genus level. **(D)** Violin plot showing Firmicutes/Bacteroidetes ratio between vitamin D deficient and normal group

* shows p<0.05).

3.7 Impact of trace element on gut microbiome

We further assessed serum levels of important trace elements (zinc, copper, and selenium) and any association with differences in gut microbiota composition. Overall, trace element deficiency had no significant impact on gut microbiota diversity or F/B (Supplementary Fig S4). However, the zinc deficient group showed a significant enrichment of the *Veillonella* genus whereas the copper and selenium deficient groups showed raised relative abundance for the *Dorea* and *Corynebacterium* genera, and the *Megamonas, RF-39* and *Monoglobus* genera, respectively (Figure 6 A-C).

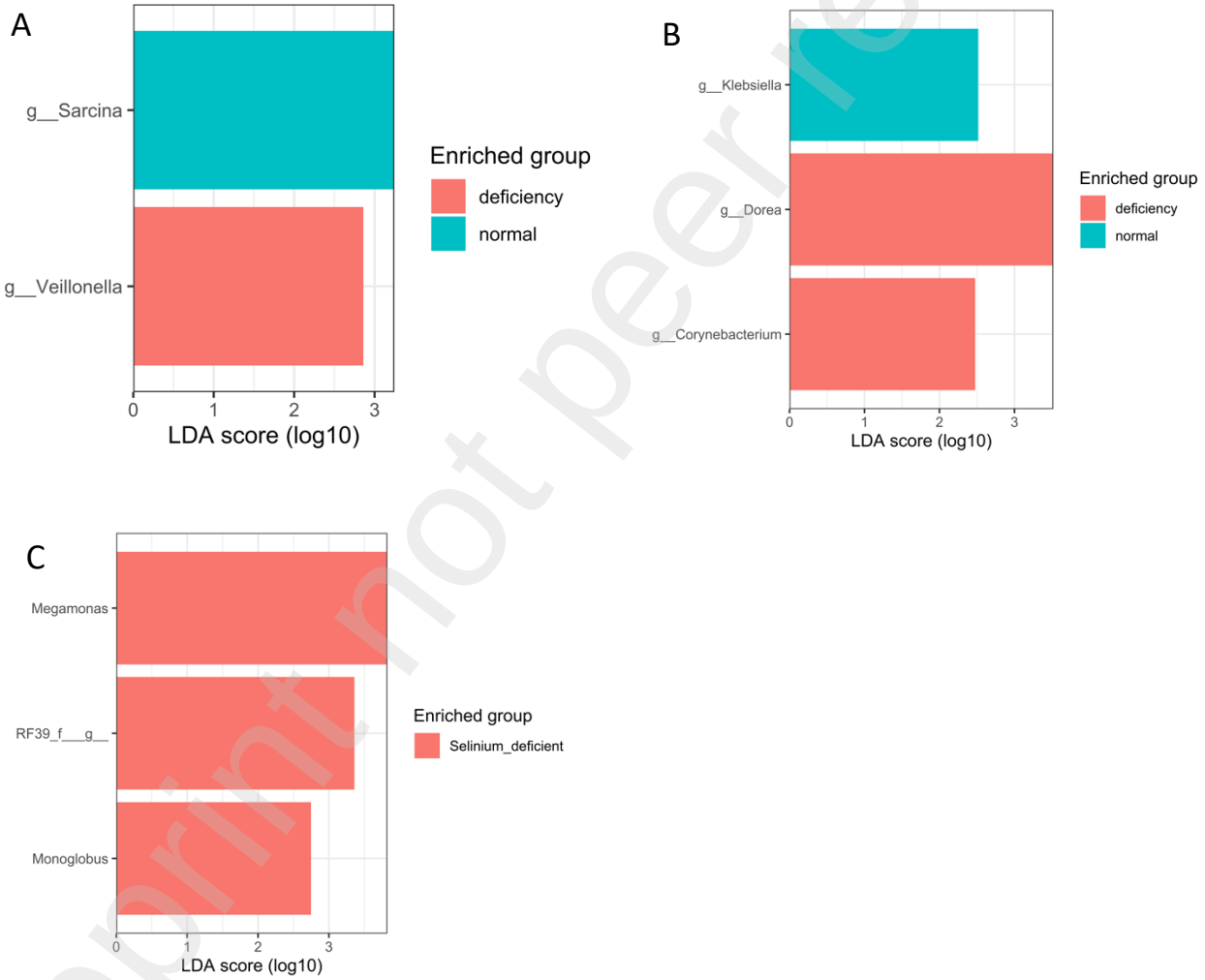

Figure :6 Linear discriminant analysis (LDA) distribution of LEfSe results based on the classification information at the genus level based on (A) Zinc deficiency **(B)** Copper deficiency & **(C)** Selenium deficiency

The threshold value of the LDA score was set to 2 and an LDA score >2 was considered significant.

3.8 Impact of multiple micronutrients deficiencies on gut microbiome

We finally examined the impact of either one (vitamin D, Vit B12, Vit B9, zinc, copper, or selenium) or multiple micronutrients deficiencies (≥2 of any of the above micronutrients) on the gut microbiome of the adolescent Afghan refugee subjects (Fig 7 A-D). Overall, the alpha diversity indices and F/B ratio were reduced in those with either single or multiple micronutrient deficiencies, although the differences were not significant. For subjects with multiple micronutrient deficiencies the LEfSe analysis also revealed enrichment of bacteria belonging to the *Coprococcus, Veillonella, Selenomonas* and *Phascolarctobacterium* genera, and depletion of the *Bacteroides* genus.

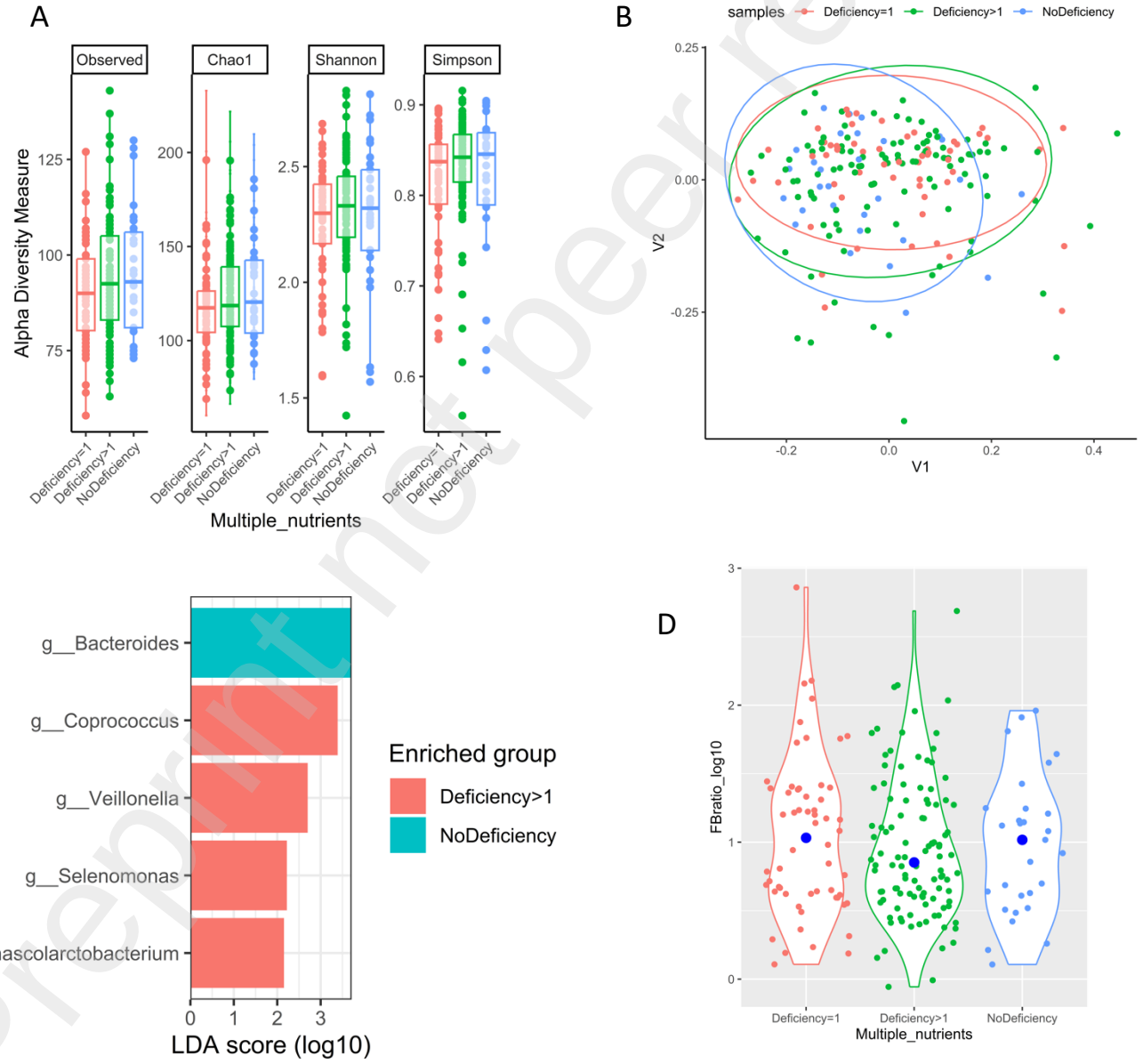

Figure 7: Bacterial diversity, composition, and abundance profile of study participants on single or multiple micronutrient deficiencies (A) Alpha diversity measures **(B)** Bray-Curtis β diversity between the normal, single, and multiple micronutrient deficiencies **(C)** Linear discriminant analysis (LDA) distribution of LEfSe results based on classification at genus level. An LDA score >2 was considered significant. **(D)** Differences in the Firmicutes/Bacteroidetes ratio between the normal, single and multiple

3.9 Correlation analysis

To further understand how the various demographic, health and nutrition status variables might impact (or be impacted by) the gut microbiota, we performed a Spearman correlation analysis (Figure 6). The F/B ratio was found to be significantly positively correlated with age and BMI, which supports the results reported above (Figs 2D & 3D). In addition, the F/B ratio was significantly negatively correlated with manganese levels (FDR < 0.05). Several bacterial taxa including 3 phyla, 6 classes, 6 orders, 10 families and 16 genera showed a significant correlation with the demographic, health and nutritional parameters. Age and BMI showed similar patterns of Spearman correlations, with 8 and 7 significant correlations, respectively, for relative abundance of specific gut microbiota taxonomic groups (Figure 8). Indeed, at phylum level, age and BMI were both significantly negatively correlated with the relative abundance of *Bacteroidetes* and positively correlated with relative abundance of *Firmicutes.* At lower taxonomic level, there were corresponding negative and positive correlations with *Prevotella* and *Dorea* from the *Bacteroidetes* and *Firmicutes* phyla, respectively (Figure 8).

With respect to micronutrients, plasma vitamin D levels were significantly negatively correlated with the *Actinobacteria* phylum (Figure 6) and this was reflected by a negative correlation with the *Bifidobacterium* genus, although this was non-significant (Figure 6). Vitamin D levels were also significantly correlated, but in a negative fashion, with the relative abundance of the *Dorea* genus (members of the *Firmicutes* phylum). Vitamin B12 was positively correlated with the relative abundance of the *Bacilli* class, and this effect is reflected at genus level where the correlation is associated with *Lactobacillus* spp.

Manganese levels were found to be correlated with more taxonomic changes than any of the other variables (13 significant correlations; Figure 8). At phylum level, there was a significant positive correlation with *Bacteroidetes* along with significant negatively correlations for *Firmicutes* and *Actinobacteria*. These phylum level changes were reflected in related significant correlations at lower taxonomic levels (e.g. positive correlations with the *Prevotella* genus from the *Bacterioidetes* phylum and negative correlation with the *Bifidobacterium* genus from the *Actinobacteria* phylum). Interestingly, the *Dialister* genus showed a significant positive correlation with manganese levels although the corresponding phylum (*Firmicutes*) displayed a negative correlation. This apparent anomaly is likely caused by compensatory negative (but non-significant) correlations for other members of the *Firmicutes* (e.g. *Clostridiaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae* and *Streptococcaceae*). Interestingly, the significant correlations for manganese are opposite to the patterns exhibited for BMI and age.

Other significant correlations were observed for: males, where there was a negative correlation with *Coriobacteriaceae* (from the *Actinomycetes* phylum); serum ferritin, which was negatively correlated with *Dialister*; copper, which was negatively correlated with *Coprococcus*; and

chromium, which displayed a negative correlation with the *Lactobacillus* genus. The negative correlation of serum ferritin with *Dialister* is of note since this contrasts with the effect seen for manganese. Indeed, the heatmap pattern for ferritin is largely opposite to that seen for manganese indicating a potential negative relationship between body-iron and manganese status (Ye et al., 2017). No significant correlations were found for folate, haemoglobin, iron, zinc or selenium.

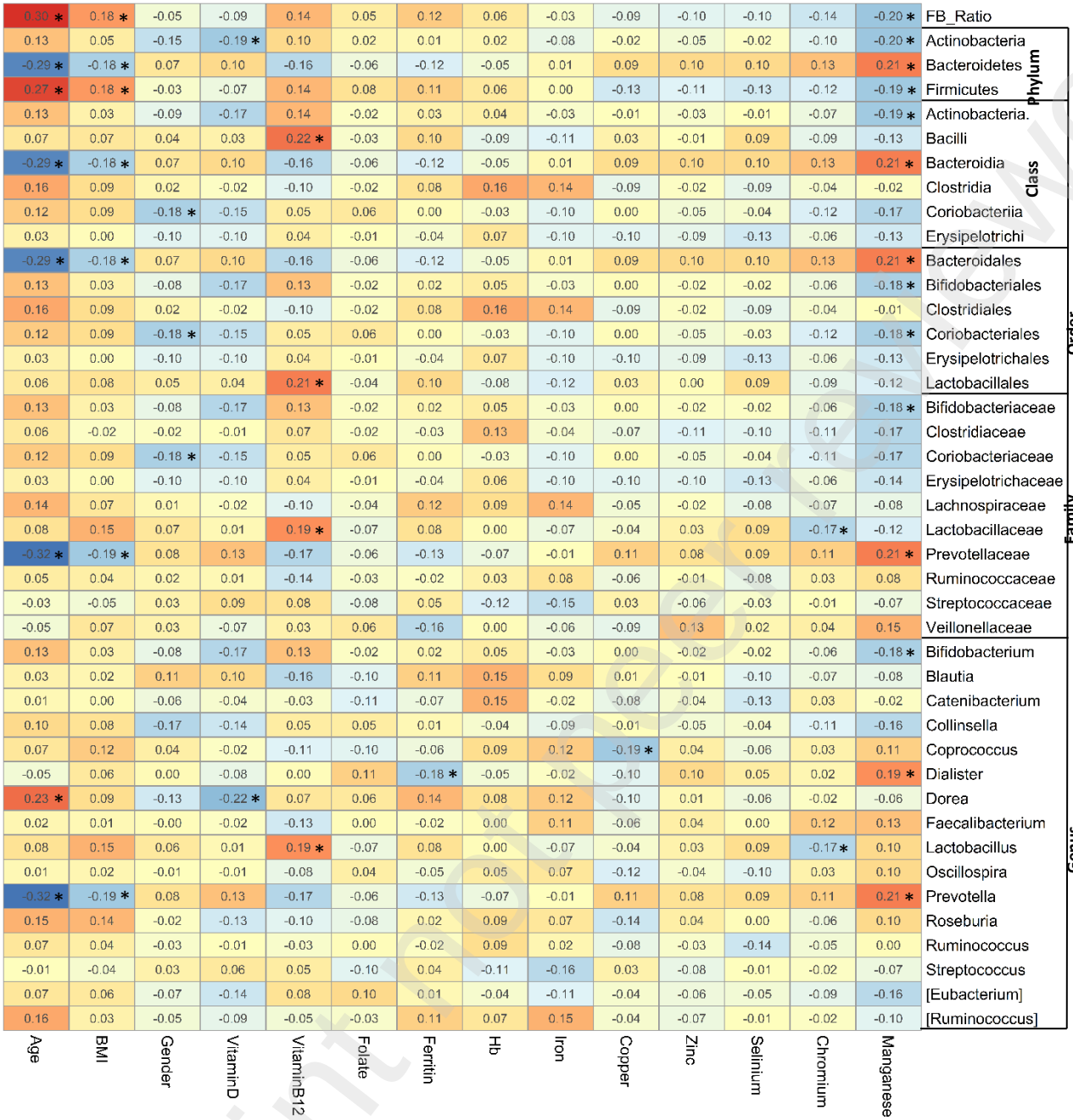

Figure 8: Heatmap of Spearman correlations between demographic, health and nutritional status variables, and the relative abundance of bacterial taxa in the gut microbiota. Data are presented as Spearman correlation coefficients. Red colour represents a positive whereas blue represents a negative correlation. Asterisks indicates false discovery rate < 0.05.

4 Discussion

During the last two decades, the human gut microbiome has been under intensive research to elucidate its role in human health and disease. However, there remains a need to explore gut microbiome dynamics in diverse and understudied populations, including major disadvantaged groups such as refugees. Therefore, we carried out this comprehensive study on a large cohort of adolescent Afghan refugees to gain insight into the relationship between their gut microbiome and demographic, health and nutritional factors. Taxonomic analysis revealed that the gut microbiome of adolescent Afghan refugees predominantly consists of bacteria belonging to phylum *Firmicutes*, followed by *Bacteroidetes* and then *Actinobacteria* as the next most abundant phyla. Although previously published research reported similar microbiome compositions (Scepanovic et al., 2019), the differences in the relative abundance of each phylum indicates that ethnicity, geography and lifestyle factors impact adolescent gut microbiota (Syromyatnikov et al., 2022).

Our study confirms and strengthens the evidence for age-related dynamics in the gut microbiome (Rinninella et al., 2019), particularly regarding *Bifidobacteria* with comparatively higher abundance in younger (10-14 years) than older (15-19 years) adolescents. *Bifidobacteria* is a large genus of beneficial saccharolytic bacteria that tend to decrease in abundance with age, though the specific timing and rate of decline can vary based on individual factors (Arboleya et al., 2016). Conversely, older adolescents in our study displayed an increase in the abundance of genus *Prevotella* and *Dialester*. These findings are in concordance with the previous reports wherein the relative abundance of genus *Prevotella* in the gut microbiota increases from childhood to adulthood (Tett et al., 2021). Moreover, Afghan refugees in Pakistan follows a traditional Afghan diet rich in carbohydrates. Since *Prevotella* species tends to dominate the gut microbiome of communities living a non-westernized, traditional lifestyle and diet, the same might also be responsible for the high relative abundance of *Prevotella* in Afghan refugee communities (Pasolli et al., 2019; Yatsunenko et al., 2012).

The *Firmicutes/Bacteroidetes* (F/B) ratio usually follows age related dynamics with higher F/B ratios in adolescents and adults compared to infants and the elderly. In the current study, an increase in the F/B ratio in the late adolescence group is consistent with previous reports wherein an increase in F/B ratio was observed with increasing age (Park et al., 2015; Vaiserman et al., 2020). Although the exact mechanism of the age-related increase in the F/B ratio is not known, it is most probably linked to the shift towards an adult-like diet and hormonal changes during puberty (Yuan et al., 2020). With respect to the BMI, the F/B ratio was slightly lower in underweight compared to normal-weight children in our study. These findings contrast with previous reports where the malnourished groups had a higher F/B ratio (Gatya et al., 2022; Méndez-Salazar et al., 2018).

Almost half of the participants in our study were classified as underweight (Table 2). Undernutrition is a widely prevalent, public health issue in low- and middle-income countries. The alarmingly high prevalence of undernutrition in Pakistan (39%) ("National Nutrition Survey 2018 - Full Report (3 Volumes) & Key Findings Report | UNICEF Pakistan," 2020) and Afghanistan (41%) ("Nutrition | UNICEF Afghanistan," n.d.), coupled with poor socioeconomic status has severe health related consequences especially in young children and adolescents. Infectious disease prevalence is usually high in malnourished children primarily due to altered immunity (Bourke et al., 2019). Malnutrition also affects gut microbiome diversity in children (Iddrisu et al., 2021). In the current study, the normal weight adolescents were found to harbor a more diverse microbiome compared to those who were underweight. These findings are in agreement with previous studies reporting a less diverse and more mature gut microbiota in 8 - 12 year old malnourished children from Indonesia compared to their healthy counterparts (Gatya et al., 2022; Vray et al., 2018). LEfSe analysis also indicated the depletion of beneficial *Lactobacillus* spp. in underweight adolescents (Fig 3). Members of the genus *Lactobacillus* are commonly known for their probiotic activities, and their depletion is implicated in a variety of human diseases including diabetes, obesity, inflammatory bowel disease and cancers (Heeney et al., 2018). Similarly, depletion of genus *Blautia* and *Dorea* from the gut microbiome of ferritin deficient individuals in our study (Fig 4) could have important health implications. Decreased abundance of these beneficial bacteria may exacerbate iron deficiency by increasing inflammation, impairing iron absorption and decreasing short chain fatty acid-mediated iron uptake pathways (Malesza et al., 2022).

This study also evaluated the impact of micronutrient status (vitamins and trace elements) on gut microbiome composition. Vitamin D deficiency was highly prevalent (n=194; 82.3%) among the adolescent Afghan refugees (Table 2) and had a pronounced association with gut microbiota composition. The overall diversity was significantly reduced in vitamin D deficient individuals. These results align with previous studies showing reduced gut microbial diversity associated with vitamin D deficiency (Bellerba et al., 2021) that can be successfully restored/increased following vitamin D supplementation (Singh et al., 2020). LefSe analysis further revealed enrichment of the opportunistic pathogen, *Peptococcus*, and a depletion of potentially beneficial *Weissella* spp. (Fig 5C). Increased abundance of *Peptococcus* has also been reported in vitamin D deficient mice which was associated with impaired glucose tolerance in adult rats and their off-spring (Liu et al., 2023). Interestingly, an 8-week supplementation of vitamin D not only reduced *Peptococcus* abundance but also improved glucose tolerance. Similar findings were also reported from a pilot study in humans wherein decreased alpha diversity and an increase in the abundance of several different bacterial species was observed in patients diagnosed with knee osteoarthritis coupled with vitamin D deficiency (Ramasamy et al., 2021). Our study also revealed some significant gut microbiota compositional changes associated with vitamin B12 deficiency including decreases in relative abundance of *Blautia,* a genus of anaerobic bacteria widely known for their probiotic

potential (Liu et al., n.d.) (Supplementry Fig S2). In population-based cross sectional studies, the abundance of *Blautia* is consistently associated with lower risk of metabolic syndrome and inflammation (Liu et al., n.d.; Ozato et al., 2019). Therefore, a depletion of these beneficial bacteria may indicate an important risk factor in our study population.

The current study also identified a significant change in abundance of specific bacterial genera in response to trace elements deficiency. Some of these bacteria are commonly implicated in different diseases and conditions affecting human health. For example, members of the *Veillonella* genus were significantly enriched in the gut microbiome of zinc deficient individuals. Zinc is an important trace element possessing anti-inflammatory and antioxidant effects in humans. *Veillonella* abundance was previously reported to be enhanced in zinc-deficiency associated conditions such inflammatory bowel disease (Rocha et al., 2023). A number of animal based, *in vivo* studies have also reported beneficial effects of zinc supplementation on the gut microbiota through the reduction of inflammation and oxidative stress (Pajarillo et al., 2021). Similarly, copper deficiency was associated with a high relative abundance of the *Corynebacterium* and *Dorea* genera, bacteria that are commonly implicated as opportunistic pathogenic infections in humans (Bernard, 2012).

An important feature of micronutrient deficiencies is that they are seldom present as a single micronutrient deficiency. In the vast majority of the cases, multiple micronutrient deficiencies exists (Semba, 2012) especially in children from low and middle income countries where such deficiencies are associated with impaired growth, morbidity and mortality. Therefore, we further categorized participants into three categories with no, single or multiple micronutrient deficiencies. A high relative abundance of four different bacterial genera was observed in participants exhibiting multiple micronutrient deficiencies. Our study findings support the results of a recent study reporting gut microbiome dysbiosis and altered energy metabolism associate with multiple micronutrient deficiencies in early life murine model (Littlejohn et al., 2023). However, deficiency of the trace elements zinc, copper, selenium either alone or in combination (multiple micronutrient deficiency) did not affect gut microbial diversity or community structure. This contrasts with previous studies reporting gut microbiota alterations associated with deficiencies in the minerals iron and zinc (Chen et al., 2022). This discrepancy might be due differences in the age of the study population as these other studies were conducted in younger children (8 years) and women of childbearing age (>30 years). In addition, deficiency of zinc and iron (and selenium) was not as common as for vitamin D in our study (Table 2) which may explain why there was no clear, significant impact of these minerals on the gut microbiome. Further, there is a possibility that the gut microbiome of the refugees studied is relatively unresponsive to micronutrient status. It may be due to isolated location, poor socioeconomic status, food insecurity and limited dietary diversity. The future studies on gut microbiome of refugee population should consider all these variables.

Study limitations

Although the current study is the first, comprehensive report on the gut microbiome and associated co-variates of adolescent Afghan refugees, it has several limitations. Firstly, the study focus was adolescents aged 10 - 19 years. We did not collect any information about early life events such as gestational age, delivery mode, breast feeding and complimentary food practices; all these factors have a significant impact on gut microbiome development (Ames et al., 2023). Secondly, due to the cross-sectional study design employed and the absence of validated food frequency questionnaires, we could not capture information about dietary intake patterns and behaviors that are related to gut microbiome diversity and development. Thirdly, the age range in the current study was broad and pubertal status may have contributed towards the inter-individual variations in the gut microbiome; this was not assessed in our study. Fourthly, the use of 16S rRNA sequencing provides limited information about the microbiome composition and function, especially at species and strain level, and our approach focused solely on the bacterial population and thus failed to consider other elements of the microbiota, e.g. fungi.

5. Conclusion

This study, for the first time, explored the gut microbiota of a cohort of healthy adolescent Afghan refugees. We have found that demographic, health, and nutrition variables could at least partly explain the gut microbiota diversity, acting as sources of variations in this rarely explored population. The gut microbiome diversity and composition were affected by age, BMI and micronutrient status. The study provides baseline gut microbiota profile and associated factors among adolescent Afghan refugees. The study findings will further guide future research to explore the role socioeconomic status, life experiences, migration and environmental factors shaping the gut microbiome and developing microbiome based, holistic approaches to improve the health of these understudied and vulnerable population.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, [SCA], upon reasonable request.

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