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Original Research

Nutritional status reshapes gut microbiota composition in adolescent Afghan refugees in Peshawar, Pakistan

Muhammad Shahzad[a,b], *Muhammad Arshad*[c], *Habab Ali Ahmad*[d], *Ishawu Iddrissu*[e], *Elizabeth H. Bailey*[f], *Nizar Dru*[c], *Shabir Khan*[b], *Haris Khan*[b], *Simon C. Andrews*[e,*]

[a] *Faculty of Dentistry, Zarqa University, Zarqa, Jordan*
[b] *Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan*
[c] *Center for Genomics and Systems Biology, New York University, Abu Dhabi, United Arab Emirates*
[d] *Department of Biological and Health Sciences, Pak-Austria Fachhochschule Institute of Applied Science and Technology (PAF-IAST), Haripur, Pakistan*
[e] *School of Biological Sciences, Health and Life Sciences Building, University of Reading, Reading, United Kingdom*
[f] *School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough, Leicestershire, United Kingdom*

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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. In this study, we hypothesized that overall nutritional status, as indicated by serum micronutrients concentrations, is an important driver of variations in gut microbiome composition. Therefore, gut-microbiome diversity and associated demographic, health and nutritional factors were assessed 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 the faecal samples. Bacterial diversity (alpha and beta diversity) and the *Firmicutes:Bacteroidetes* (F/B) ratio were significantly higher in the 15 to 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 micronutrient (vitamins and minerals) status. These results were further scrutinised by correlation analysis which confirmed that age, BMI and micronutrient status

* *Corresponding author at*: School of Biological Sciences, University of Reading, RG6 6EX, Reading, United Kingdom
E-mail address: s.c.andrews@reading.ac.uk (S.C. Andrews).

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show significant correlations with F/B ratio and the relative abundance of specific bacterial taxa. Collectively, our study provides the first indication of how the gut-microbiota profile of adolescent Afghan refugees is associated with a range of nutrition-status factors. These findings can thus provide a basis for translational microbiota research aimed at improving the health of such understudied and vulnerable populations.

1. Introduction

The human gut contains trillions of microbes (bacteria, viruses, fungi and archaea) that live in a mutually-beneficial, commensal relationship with the host and together constitute the gut microbiota [1]. The human host offers the microbiota a nutrient-rich and stable environment, while the gut microbiota provides essential nutrients (e.g. vitamins) that support host metabolism [2], and modulates immunity [3] and host health (both physical and mental) [4,5]. Although the gut microbiome is relatively stable in healthy adults, disturbance in its composition, known as microbial dysbiosis, is linked to a variety of human pathologies including inflammatory bowel disease, colorectal cancer, obesity, allergy and autoimmune diseases [5].

A growing body of research indicates that there are variations in gut microbiota diversity and composition between different human population groups [6]. These variations are influenced by a range of factors that are broadly categorized into three types: geographical, ecological and host related. Until now, most research has focused on host-related factors such as genetics, ethnicity, diet and lifestyle, while the role of geographic location combined with relocation (human migration) has received relatively little attention. Geographic location has been identified as an important factor affecting the diversity of the gut microbiome ecosystem [7]. A recent study [8] 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 [8].

Geographic relocation, including cross-border movements, forced migration and refugeeism, offers a unique perspective on the impact of environment and lifestyle on the human microbiome. The 2022 report of the United Nations High Commission for Refugees reported that, globally, 108.4 million people were forcibly displaced [9]. Of these, 35.3 million were 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 [10]. The majority of those in refugee camps suffer from food insecurity, unhygienic conditions, poverty, psychological stress, pollution and limited access to healthcare provision [11]. As a result, refugee populations are highly vulnerable to both communicable and non-communicable diseases, and the resultant detrimental health consequences [12]. 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 from Syria and Ukraine [9]. Following the Soviet invasion of Afghanistan in 1978 to 1979, 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 nonregistered (illegal) Afghan refugees [13]. 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 [14]. We hypothesized that this high prevalence of malnutrition will result in alterations in gut microbiome diversity and that such changes will correlate with nutritional status. However, till now, no research study has evaluated the gut microbiome composition of this population, despite the potential influence on health [15]. In the current study, we have characterized the gut microbiome and its functional potential in adolescent Afghan refugees residing in a refugee camp in Peshawar (Pakistan). In addition, stratification of the data is provided according to body mass index (BMI), nutritional status and micronutrient profile. It should be noted that the metagenomic data of this study was previously made available (https://www.ncbi.nlm.nih.gov/sra/PRJNA1105775; [16]).

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 [14], a total of 206 participants (103 male and 103 female) fulfilling the following inclusion criteria were recruited: (a) healthy adolescent children, aged 10 to 19 years (age consistent with the WHO definition of adolescence) based on physical examination indicating the absence of chronic diseases, as confirmed

by medical screening by a trained physician; (b) not taking any nutritional or micronutrient supplement; and (c) living in the study area for at least one year. Participants were divided into two age groups: 10 to 14 and 15 to 19 years, based on developmental stages of early and late adolescence, which correspond to significant physiological and hormonal changes that may influence gut microbiota composition. Further details regarding study population are provided elsewhere [14]. 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 antidiarrhoea 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 was collected using an interviewer-administered questionnaire. Anthropometric measurements, including height and weight, were recorded following standard protocols. Height was measured using a wall-mounted stadiometer and weight was measured on an electronic scale (Secca, UK). Measurements were taken three times, and the average was used to calculate body mass index (BMI).

2.3. *Collection of biological samples*

Blood and faecal samples were collected from all participants. Nonfasting, whole-blood samples were collected by a trained phlebotomist in the morning using standard procedures. The samples were processed and frozen at −80 °C. Participants were provided with a stool sample collection kit and instructed to return the sample within one hour of defecation. Samples were immediately mixed with an equal volume of DNA/RNA shield (Zymo Research, USA) for stabilization and transported to the laboratory under temperature-controlled conditions.

2.4. *Laboratory analysis*

Blood parameters were analyzed using an automated haematology analyser (Sysmex XP-100, Singapore) for complete blood counts and for haemoglobin, haematocrit and mean corpuscular volume assays. Ferritin, vitamin B12 and folate were measured using an Abbott Architect i2000 (Abbott Diagnostics, Switzerland). Vitamin D concentrations were assessed using a Diasorin ELISA kit (Euroimmun, Germany) with a random error of ±5%. The cutoff values for vitamin D, ferritin, vitamin B12 and folate deficiency were <20 ng/mL, <15 ng/mL, <203 pg/mL and <3 ng/mL, respectively, consistent with WHO guidelines.

Elemental concentrations of zinc, copper, selenium and iron were measured using ICP-MS (Thermo Fisher Scientific iCAPQ, Germany) with samples processed through an ASXpress^{TM} module (Cetac ASX-520, USA) and PFA-ST nebulizer. Reference ranges for the measured elements were: zinc (70-120 µg/dL), copper (80-155 µg/dL), selenium (60-140 µg/dL) and iron (50-170 µg/dL). All analyses were performed following manufacturers' protocols. Briefly, all samples and external multielement 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). 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^{TM} L-1, Lot 1801802, and Seronorm^{TM} L-2, Lot 1801803; Nycomed Pharma AS, Billingstad, Norway); these were run at the start and the end of sample batch runs and the average recoveries recorded for both

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 (the bead-beating step was repeated once and DNA elution was performed in two steps using 0.03 mL of distilled water per step, incubating at room temperature for 1 minute before centrifugation at 13,000 g for 1 minute [17]). Samples were defrosted, washed and resuspended in InhibitEX buffer. Glass beads were added and the samples were bead-beaten three times. The supernatant was treated with Proteinase K and AL buffer, then loaded onto QIAamp spin columns. After washing, the DNA was eluted and stored at −20 °C until submission on dry ice for NGS. DNA quality was assessed by agarose gel electrophoresis, Nanodrop analysis and PCR amplification. 16S rRNA gene amplicon-based sequencing was performed by the 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 <.05 was considered significant.

2.6.2. *Bioinformatics data analysis*

The 16S rRNA gene sequencing data were analyzed with DADA2 [18] software to determine sequencing error rates, to dereplicate amplicons and to remove chimeric sequences.

Reads were trimmed (forward: 280 bp, reverse: 160 bp) to ensure high quality (phred >30), with filtering parameters maxN = 0, maxEE = 2 and truncQ = 2 (clean reads data: Supplemental Table 1). Taxonomy assignment was performed using Kraken2 (v2.1.2) with abundance estimation by Bracken (v2.8) [19] at multiple taxonomic levels (threshold = 5) using the Greengenes Database (v13.5) (Lu & Salzberg, 2020). Read count data obtained from the Kraken2 analysis assessed alpha diversity differences between groups via Wilcox test ($P < .05$). Beta diversity was analyzed using multidimensional scaling (MDS) plots based on Bray-Curtis dissimilarity matrices (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 [20]. PICRUSt2 results were visualized with ggpicrust2 (v1.7.2) vignette [21]. 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 1. The mean age of the study participants was 13.4 ± 2.9 years with no significant differences between the female and male groups. The majority (n = 126; 64.3%) of the participants were in the age range 10 to 14 years, living in families 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 and multiple micronutrients deficiencies were also common (vitamin D deficiency, 82.3%; vitamin B12 deficiency, 43%; folate deficiency, 70%; selenium deficiency, 50%; zinc deficiency, 35.6%).

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 read statistics are presented in Supplemental 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 the 196 faecal samples. The *Firmicutes* (82%), *Bacteroidetes* (13.2%) and *Actinobacteria* (2.9%) were the most abundant bacterial phyla overall, contributing 98% of the reads (Supplemental Fig. S1A). *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 (Supplemental Fig. S1C).

3.3. Impact of age and gender on the gut microbiota

We next assessed age and gender-based differences in the gut microbiome of the sample population. As shown in Fig. 1A, the alpha diversity measures, assessing species richness and evenness (Observed, Chao1, Shannon and Simpson), were higher in the 15 to 19 age group compared to 10 to 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 (MDS) of the beta diversity analysis (Fig 1B). To further differentiate taxonomic differences between the gut microbiota of the two age groups, we used linear discriminant analysis (LDA) effect size (LEfSe) which indicates the effect size of each of the abundant genera (Fig 1C). The gut microbiomes of the 10 to 14 age group showed significant enrichment of 11 bacterial genera with *Prevotella_9*, *Prevotella* and *Dialester* being the top three most abundant genera affected. In the 15 to 19 age group, *Bifidobacterium*, *Dorea* and *Romboutsia* were the top three enriched genera. The *Firmicutes* to *Bacteroidetes* ratio (F/B ratio), the most widely used indicator of gut microbial dysbiosis [22], was significantly higher in the 15 to 19 age group compared to 10 to 14 age group (Fig. 1D).

No overall significant gender-based differences in gut microbiota alpha or beta diversity, or F/B ratio, were observed. 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 (Supplemental Fig. S2A-D).

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 group than the underweight group. However, the differences in diversity indices between the two groups were only significant for the Shannon index (Fig. 2A). The NMD beta diversity analysis showed no apparent stratification of gut microbiome composition based on BMI (Fig. 2B). 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. 2C). The F/B ratio was also slightly higher in the normal weight individuals, although this difference was not significant (Fig. 2D).

Table 1 – Sociodemographic characteristics and micronutrients status of adolescent Afghan refugees (n = 196).

Characteristics		Total n (%)	Gender		
			Male n (%)	Female n (%)	P-value[a]
Age categories	10-14	126 (64.3)	67 (71.3)	59 (57.8)	.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 and above	19 (9.7)	7 (7.5)	12 (11.8)	
Education Categories	No formal education	39 (20)	8 (8.6)	31 (15.9)	.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 and university	2 (1)	2 (2.2)	0 (0)	
Income categories	<25,000 PKR	143 (73.3)	66 (71)	77 (75.5)	NS
	≥ 25,000 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)	.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)	.025
	No	170 (90.4)	87 (95.6)	83 (85.6)	
Vitamin B12 deficiency based on WHO criteria of <203 pg/mL)	No	104 (57.1)	39 (44.8)	65 (68.4)	.002
	Yes	78 (42.9)	48 (55.2)	30 (31.6)	
Folate concentration	Normal (6-20 ng/mL)	53 (27)	15 (16)	38 (19.4)	.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 concentration	Normal	141 (73.8)	68 (74.7)	73 (73)	NS
	Deficiency	50 (26.2)	23 (25.3)	27 (27)	
Zinc concentration	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)	.001
	Deficiency	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)	

[a] The Chi-square test was used to calculate differences between the male and female groups.NS, nonsignificant; PKR, Pakistani rupees.

3.5. *Association between iron status and the gut microbiome*

Serum concentrations 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 concentration suggestive of deficient body iron stores (Table 1). Observed richness, diversity and composition were not significantly different between anaemic and nonanaemic participants (Supplemental Fig. S3A-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 (Supplemental Fig. S3C). Nonsignificant differences in alpha and beta diversity were also observed between the participants with normal and low serum ferritin concentrations (Supplemental Fig. S3DE). In addition, the gut microbiomes of individuals with low serum ferritin concentrations showed a significantly lower abundance (LDA score >2; $P < 0.05$) of six genera (*Blautia*, *Dorea*, *Butyricicoccus*, *Sarcins*, *Fusicatenibacter*

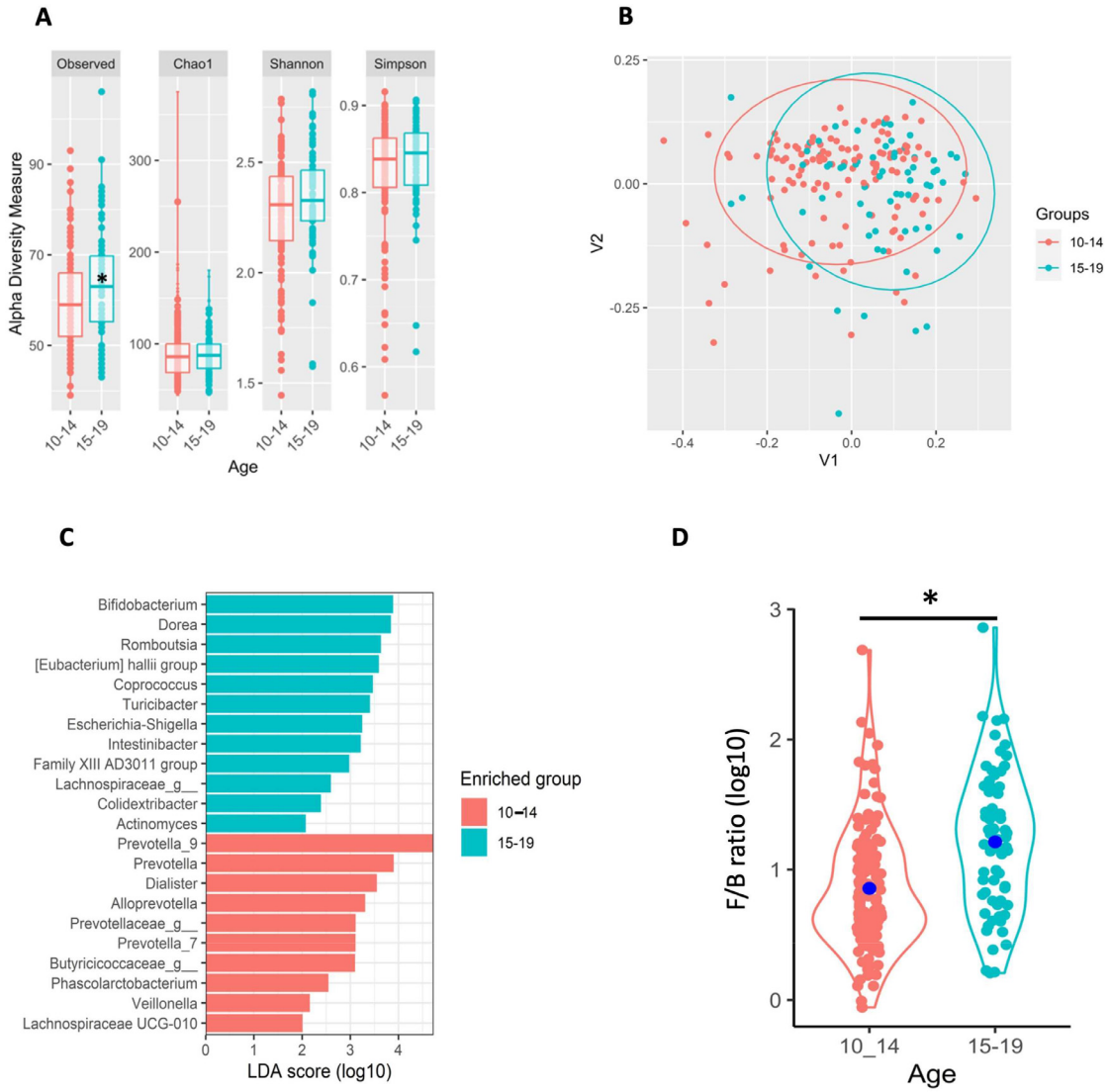

Fig. 1 – Taxonomic diversity, composition and abundance profiling of the study participants based on age groups. (A) Alpha diversity measures were higher in the 15 to 19 age group compared to 10 to 14 age group (* shows statistical significance between age groups at $P < 0.05$). (B) Bray-Curtis β diversity showed significant differences ($P = 0.006$) based on MDS between the two age groups. (C) 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. The gut microbiomes of the 10 to 14 age group showed significant enrichment of 11 bacterial genera. (D) Violin plot showing F/B ratio between the two age groups (* shows $P < 0.05$). MDS, multidimensional scaling; LDA, linear discriminant analysis; LEfSe, LDA effect size; F/B, *Firmicutes/Bacteroidetes*

and *Klebsiella*) along with significant enrichment of the *Alloprevotella*, *Catenisphaera* and *Oribacterium* genera (Supplemental Fig. S3F)

3.6. *Impact of vitamin concentrations on gut microbiome*

Next, we assessed the relationship/association between gut microbiota composition and serum vitamin D, vitamin B9 and vitamin B12 concentrations in the adolescent Afghan refugees. Vitamin D status showed an impact on gut microbiota as indicated by a significantly lower Chao1 diversity index (Supplemental Fig. S4A) and enrichment of *Peptococcus* and *Methanobrevibacter* genera, and depletion of the *Weissella* genus (Supplemental Fig. S4C) in vitamin D deficient individuals compared to the sufficient group. Serum vitamin D concentrations had no significant impact on beta diversity or F/B ratio (Supplemental Fig. S4B and D). Similarly, gut microbiota alpha- and beta-diversity indices and F/B ratios were not significantly different between the normal and either the vitamin B12 (cobalamin) or vitamin B9 deficient groups (Supplemental Fig. S5 and S6). However, significant differences were observed in 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 (Supplemental Fig. S5). In addition, the *Shuttleworthia*, *Phasecolarctobacterium* and *Bullei-*

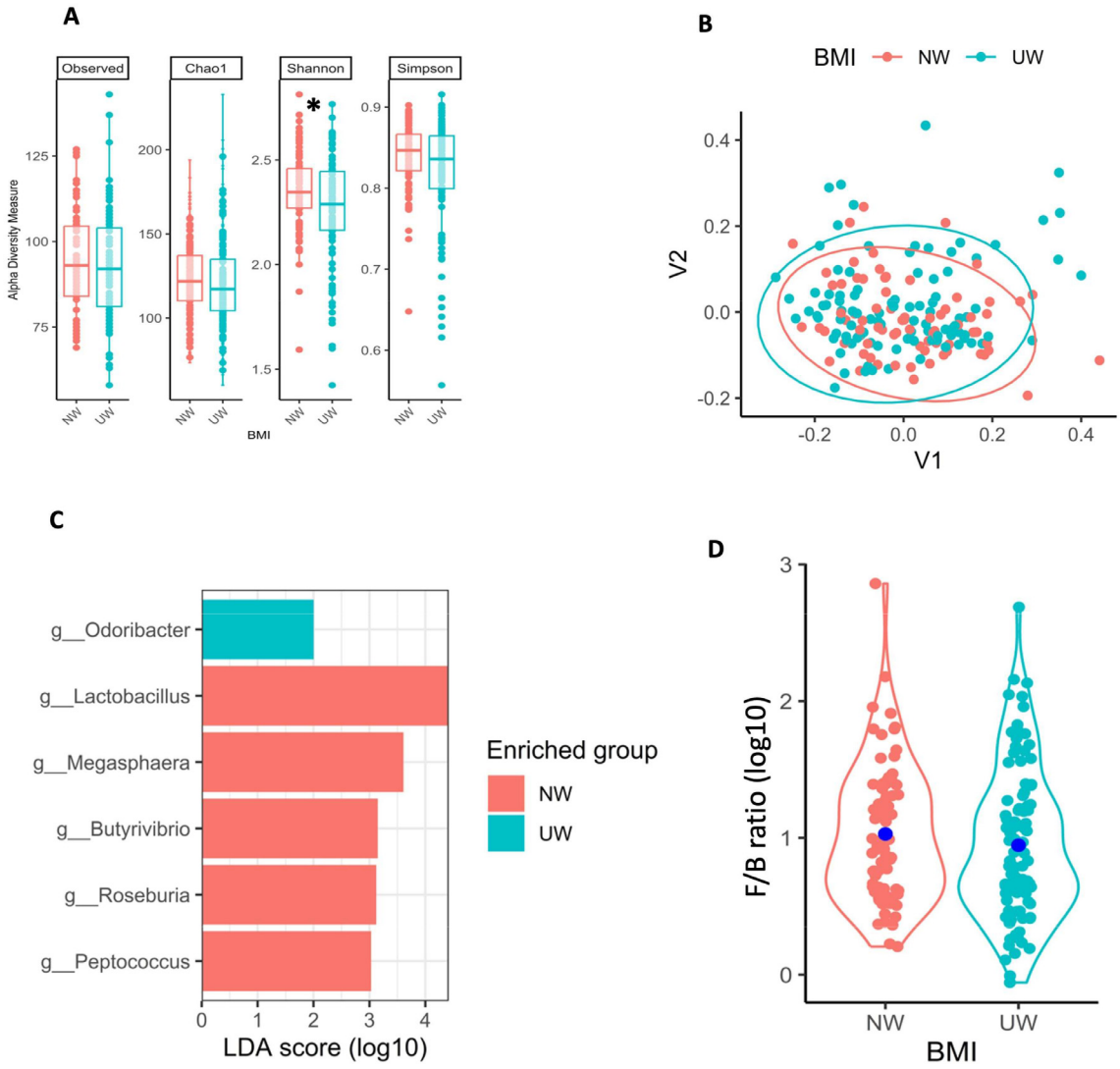

Fig. 2 – Bacterial diversity, composition and abundance profile of study participants on the basis of BMI. (A) Alpha diversity measures (*, significant difference $P < 0.05$) were higher in the normal-weight than the underweight group. (B) There was no significant difference in β-diversity based on Bray-Curtis dissimilarity between the normal-weight and underweight adolescents. (C) LEfSe results based on classification at genus level. An LDA score of >2 was considered significant. (D) The F/B ratio was also slightly higher in the normal weight individuals. LEfSe, linear discriminant analysis effect size; F/B, Firmicutes:Bacteroidetes

dia genera were highly enriched in the folate deficient group (Supplemental Fig. S6).

3.7. *Impact of trace element on gut microbiome*

We also assessed the serum concentrations 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 ratio (Supplemental Fig. S7). 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 (Supplemental Fig. S7A-C).

3.8. *Impact of multiple micronutrients deficiencies on gut microbiome*

We finally examined the impact of either one (vitamin D, vitamin B12, vitamin 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 participants (Supplemental Fig. S8A-D). Overall, the alpha diversity indices and F/B were not significantly different. For those with multiple micronutrient deficiencies, the LEfSe analysis revealed enrichment of bacteria belonging to the

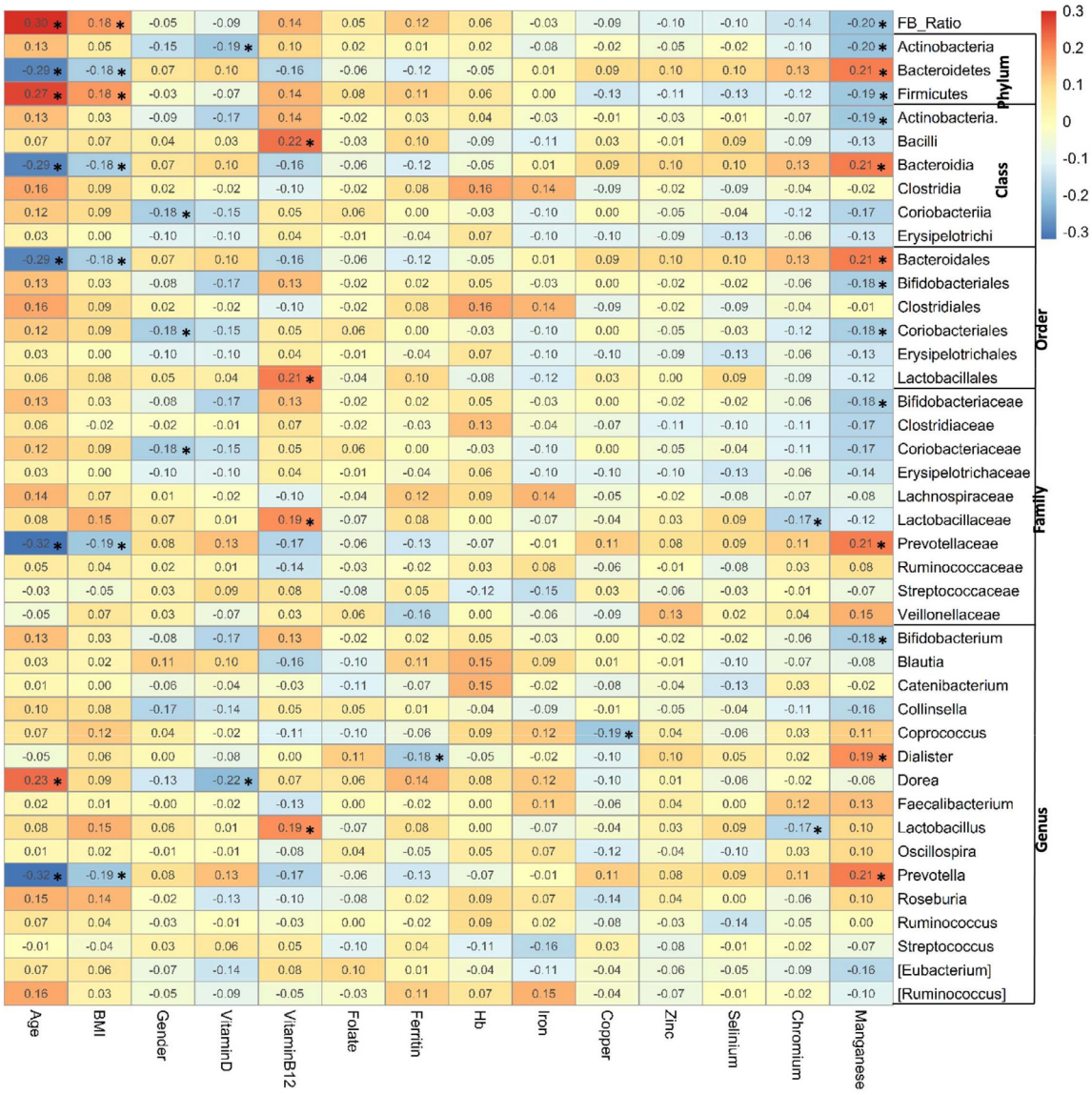

Fig. 3 – Heatmap of Spearman correlations between demographic, health and nutritional status variables, and the relative abundance of bacterial taxa in the gut microbiota. Data are presented as Spearman correlation coefficients. Red and blue colours represent a positive and negative correlation, respectively. Asterisks indicates FDR < 0.05. The F/B ratio is significantly positively correlated with age and BMI, and negatively correlated with manganese concentration (FDR < 0.05). Several bacterial taxa (3 phyla, 6 classes, 6 orders, 10 families and 16 genera) showed a significant correlation with the demographic, health and nutritional parameters. FDR, false discovery rate; F/B, Firmicutes:Bacteroidetes ratio; BMI, body mass index.

Coprococcus, *Veillonella*, *Selenomonas* and *Phascolarctobacterium* genera, and depletion of the *Bacteroides* genus.

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, a Spearman correlation analysis was performed (selected due to the nonparametric distribution of the data) (Fig. 3). The F/B ratio was found to be significantly positively correlated with age and BMI, which supports the results reported above (Figs. 1D and 2D). In addition, the F/B ratio was significantly negatively correlated with manganese concentrations (FDR < 0.05). Several bacterial taxa (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 (Fig. 3). 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 (Fig. 3).

With respect to micronutrients, plasma vitamin D concentrations were significantly negatively correlated with the relative abundance of *Actinobacteria* phylum members (Fig. 3) and this was reflected by a negative correlation with the *Bifidobacterium* genus, although this was nonsignificant (Fig. 3). Vitamin D concentrations 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 was reflected at genus level where the correlation is associated with *Lactobacillus* spp.

Manganese concentrations were found to be correlated with more taxonomic changes than any of the other variables (13 significant correlations; Fig. 3). At phylum level, there was a significant positive correlation with *Bacteroidetes* along with significant negative correlations for *Firmicutes* and *Actinobacteria*. These phylum level changes were reflected in related significant correlations at lower taxonomic levels (e.g. positive correlation 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 concentrations although the corresponding phylum (*Firmicutes*) displayed a negative correlation. This apparent anomaly is likely caused by compensatory negative (but nonsignificant) 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: there was a negative correlation with *Coriobacteriaceae* (from the *Actinomycetes* phylum) relative abundance for males with respect to females; serum ferritin was negatively correlated with *Dialister* abundance; copper was negatively correlated with *Coprococcus* abundance; and chromium 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 [23]. No significant correlations were found for folate, haemoglobin, iron, zinc or selenium.

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 the *Firmicutes* phylum, followed by *Bacteroidetes* and then *Actinobacteria* as the next most abundant phyla. Previously published research reported similar microbiome compositions [24]. Häsler et al. [25] found that the faecal microbiota of refugees from Syria, Iraq and Afghanistan differs significantly from that of Germans residing in Germany. They found that *Firmicutes* and *Actinobacteria* are more common in the German population. On the other hand, *Proteobacteria*, including potential pathogens such as *Klebsiella pneumoniae, Haemophilus influenzae* and *Shigella sonnei,* and *Bacteroidetes*, were significantly elevated among Afghan refugees. Additionally, *Bacteroidetes* were also associated with pathogenicity and antibiotic use in refugees [26]. The differences in the relative abundance of each phylum indicates that ethnicity, geography and nutritional status impact adolescent gut microbiota which supports our hypothesis [27].

Our study confirms and strengthens the evidence for age-related dynamics in the gut microbiome [28], particularly regarding *Bifidobacteria* with comparatively higher abundance in younger (10-14 years) than older (15-19 years) adolescents. The *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 [29]. Conversely, older adolescents in our study displayed an increase in the abundance of the *Prevotella* and *Dialester* genera. These findings are in concordance with previous reports wherein the relative abundance of the *Prevotella* genus in the gut microbiota increased from childhood to adulthood [30]. Moreover, Afghan refugees in Pakistan follow a traditional Afghan diet which is rich in carbohydrates and prebiotic fibers known to support the growth of beneficial bacteria like *Prevotella* species. Since *Prevotella* species tend- to dominate the gut microbiome of communities living a non-westernized, traditional lifestyle and diet, the same factors might also be responsible for the high relative abundance of *Prevotella* in Afghan refugee communities [27].

The F/B ratio is a widely used indicator of gut microbial health, as it follows age-related dynamics with higher F/B ratios in adolescents and adults compared to infants and the elderly [31]. However, its relevance is debated due to inconsistent associations with health outcomes. Also the exact mechanism of the age-related increases in the F/B ratio is still unknown, although it is linked to the shift towards an adult-like diet and hormonal changes during puberty [32]. Our results align with prior studies showing age-related increases, but due to the limitations in functional insights, interpreting these results requires caution. 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 that observed a higher F/B ratio in malnourished groups [33,34].

Almost half of the participants in our study were classified as underweight (Table 1). Undernutrition is a widely prevalent, public health issue in low- and middle-income countries. The alarmingly high prevalence of undernutrition in Pakistan (39%) [35] and Afghanistan (41%) [36], 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 impaired immunity [37]. Malnutrition also af-

fects gut microbiome diversity in children [38]. 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 to 12 year old malnourished children from Indonesia compared to their healthy counterparts [33,39]. The low prevalence of anemia in this population, despite widespread undernutrition, may be explained by iron-fortification initiatives in Pakistan. Although not mandatory, efforts to enrich wheat flour with iron at local mills may have contributed to sufficient iron status. LEfSe analysis also indicated the depletion of beneficial *Lactobacillus* spp. in underweight adolescents (Fig. 2). 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 [40]. Similarly, depletion of the *Blautia* and *Dorea* genera from the gut microbiome of ferritin-deficient individuals in our study (Supplemental Fig. 4F) 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 [41].

Physiological effects have been associated with alterations in gut microbial composition. For example, dietary-fiber-enriched diets selectively promote beneficial bacteria such as *Akkermansia muciniphila* and SCFA-producing bacteria, which have been shown to improve glucose homeostasis, reduce HbA1c concentration and improve insulin sensitivity in type 2 diabetes patients [42]. High fat diets induce distinct microbial profiles and promote proinflammatory cytokines, such as IL-17 and IFN-γ, which increases systemic inflammation and adaptive immune activation [43], while taxa associated with lean diets support regulatory immune pathways and metabolic stability. In addition, a lower abundance of *Faecalibacterium prausnitzii*, a butyrate-producing bacterium that has anti-inflammatory properties, is seen in patients with IBD, which could contribute to worsening of intestinal inflammation and impairment of gut barrier function [44]. These examples illustrate the importance of specific microbial taxa in controlling metabolic, immune and inflammatory processes.

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 1) likely due to limited sun exposure influenced by the geographic latitude (34° N for Peshawar) and cultural clothing practices such as full-body covering. Participants with vitamin D deficiency showed significant changes in microbial diversity. These results align with previous studies showing reduced gut microbial diversity associated with vitamin D deficiency [45] that can be successfully restored/increased following vitamin D supplementation [46]. LefSe analysis further revealed enrichment of the opportunistic pathogen, *Peptococcus*, and a depletion of potentially beneficial *Weissella* spp. (Supplemental Fig. S4). 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 [47]. 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 where decreased alpha diversity and an increase in the abundance of several different bacterial species were observed in patients diagnosed with knee osteoarthritis coupled with vitamin D deficiency [48]. Our study also revealed some significant gut microbiota compositional changes associated with vitamin B12 deficiency including decreases in the relative abundance of *Blautia*, a genus of anaerobic bacteria widely known for their probiotic potential [49] (Supplemental Fig. S5). In population-based cross sectional studies, the abundance of *Blautia* is consistently associated with lower risk of metabolic syndrome and inflammation [49]. 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 element deficiency. Some of these bacteria are commonly implicated in various 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 that has anti-inflammatory and antioxidant effects in humans. *Veillonella* abundance was previously reported to be enhanced in zinc-deficiency associated conditions such as inflammatory bowel disease [50]. 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 [51]. Similarly, copper deficiency was found to be associated with a high relative abundance of the *Corynebacterium* and *Dorea* genera, bacteria that are commonly implicated as opportunistic pathogens in humans [52].

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 [53], 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 (including zinc, folate/vitamin B9, iron, vitamin A and vitamin B12) for adolescent individuals using an early life murine model [54]. However, deficiency of the trace elements zinc, copper and 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 [55]. 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 1) which may explain why there was no clear, significant impact of these minerals on the gut microbiome. While significant correla-

tions were observed between nutritional variables and microbiota composition, the role of environmental factors, such as diet, cannot be ignored. The dietary patterns in the refugee camp primarily consist of starchy staples like wheat, barley, rice, lentils and chickpeas, which are rich in prebiotic fibers that influence gut microbiota. Further, there is a possibility that the gut microbiome of the refugees studied is relatively unresponsive to micronutrient status due to factors such as isolated location, poor socioeconomic status, food insecurity and limited dietary diversity. Future studies on the gut microbiome of refugee population should address such variables.

Although the current study is the first, comprehensive report on the gut microbiome and associated co-variates in adolescent Afghan refugees, it has several limitations. Firstly, the study focus was adolescents aged 10 to 19 years. We did not collect any information about early life events such as gestational age, delivery mode, breast feeding and complementary food practices; all these factors have a significant impact on gut microbiome development. 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, we have used Greengenes 2020 database for 16S rRNA analysis. While this database is one of the most widely used resources for taxonomic profiling, it should be noted that this version (v13.5) might not provide the most recent taxonomic updates available in current databases. Finally, 16S rRNA sequencing provides limited detail on 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.

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 a baseline gut microbiota profile, and associated factors, among adolescent Afghan refugees. Additionally, the findings will help guide future research exploring the role of socioeconomic status, life experiences, migration and environmental factors in shaping the gut microbiome, and will assist in developing microbiome-based, holistic approaches for improving the health of such understudied and vulnerable populations.

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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.

Author declarations

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nutres.2025.04.004.

CRediT authorship contribution statement

Muhammad Shahzad: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Data curation, Conceptualization. **Muhammad Arshad:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Habab Ali Ahmad:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation. **Ishawu Iddrissu:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Elizabeth H. Bailey:** Writing – original draft, Methodology, Investigation, Formal analysis. **Nizar Dru:** Writing – original draft, Investigation, Formal analysis, Data curation. **Shabir Khan:** Writing – original draft, Methodology, Investigation, Data curation. **Haris Khan:** Writing – original draft, Methodology, Investigation, Data curation. **Simon C. Andrews:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

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