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RESEARCH ARTICLE

COMPARISON OF MICROBIOTA AMONG HEALTHY CHINESE AND FOREIGN STUDENTS

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ABSTRACT

Background: To human health, the significance of Gut Microbiota is highly cherished. The core goal of this research was to do the comparison between the gut microbiota of healthy Chinese and foreign students in Shandong, China.
Methods: Fecal samples were collected from 11 healthy Chinese students and from 31 healthy foreign students in Jinan city, Shandong. DNA was extracted, and 456 bp segments comprising hypervariable regions 3 and 4 of the 16S rRNA gene were amplified, barcoded and sequenced.
Results: A total of 1,599,407 good quality reads were obtained for evaluating bacterial diversity. *Firmicutes* was the highest abundant phylum trailed by *Bacteriodetes* and *Actinobacteria*. *Clostridium* constituted the most abundant genus in two groups, but its ratio was higher in foreign students group than Chinese students. The genera *Prevotella* was in high ratio in foreign students group and genera *bacteriodes* was in higher ratio in Chinese students group. Although clearvariation between individuals were detected, a huge amount of phylotype is still shared in each group by the healthy microbiota, signifying that a principal microbiome is present in each healthy habitat. However, the diversity and richness showed no difference, the structure of gut microbiota was significantly different between Chinese and foreign students.
Conclusion: Phylum *Firmicutes* and genera *Prevotella* and *Bacteroides* constituted the bulk of the human gut microbiota, while there were prominent alterations in composition between the two groups probably because of place, diet and lifestyle.

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INTRODUCTION

A very complex and diverse microbial community has been harbored by human intestinal tract playing a key role in Human health. There are around 100 trillion microbial cells and 100 times more genes in our gut in comparison to our Human Genome (Ley *et al*., 2006). There is huge number of viruses in and on us as well (Haynes and Rohwer, 2011). Altogether our microbiota has been constituted by the microbes that are living in the human body and on it as well, and our microbiome has been formed by the genes that encode them (Clemente *et al*., 2012). Generally, this community has been mentioned to be unseen metabolic organ due to their huge effect on wellbeing of human, which includes host's metabolism, their nutrition, immune function and physiology as well. It's clear now that microbiome of our gut coexist with us (Ley *et al*., 2008) and that any kind of disruption to them can lead to main problems, that can have both positive and damaging effects on human health (Guinane and Cotter, 2013). Till today though the number the Bacterial phyla present in the human gut has reached to more than 50 (Schloss and Handelsman, 2004), solitary 2 phyla are dominating the gut microbiota, the *Firmicutes* and *Bacteroidetes*. Different studies have shown different results on the overall amount of bacterial species existing in the gut of human, but its normally believed that around 1000 bacterial, species level phylotypes are harbored by the individuals (Claesson *et al*., 2009; Lozupone *et al*., 2012). The 'Last revealed part' of the human body is proved to be the human gut microbiota (O'Hara and Shanahan, 2006), which varies in function from digesting the food and providing protection against harmful microbes to immunity and central nervous system regulation. Diet, lifestyle, mode of delivery, medical treatment, genetics and many other factors affect its composition (Lagier *et al*., 2012). For better understanding that how human gut microbiota play a role in health and for determining new ideas to change the microbial population for the inhibition of many diseases and their treatments, it's very

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important to elucidate the global microbial diversity. Using different methods of culturing and DNA sequencing have provide better vision into the entire genomic structure of samples from metabolic and taxonomic perceptions. Now a days lot of work has been carried out on human gut microbiota all over the world which had provided huge number of metagenomic data sets of this population (Lagier *et al*., 2012). We conducted this study to have an illustrative analysis of human gut microbiota of students coming from different countries against Chinese students using 16S rRNA sequencing.

MATERIALS AND METHODS

Subjects and collection of samples

This research was conducted in Jinan city of Shandong province in year 2017. Participants included 31 healthy foreign students and 11 healthy Chinese students. Permission was taken from all the students before collecting the samples from them. Participants were chosen from independently living individuals aged 18-30 year who were in apparent good health and were not known to have any disease. Individuals who had taken any medication in the previous three months were excluded from the study. Participants were included as a sample of convenience. The samples were provided by the students at different times. The containers having the stool samples were delivered in the short time of two hours after being provided by the students. The samples were kept at -80°C to be processed in batches for the extraction of DNA. Ethics Committee of Qilu Hospital, Shandong University has permitted this research.

Extraction of DNA and PCR amplification

From fecal samples microbial DNA were extracted bythe E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the instruction of manufacturer. After that, DNA concentration and the final purification were determined by Nano Drop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the DNA quality was checked viaelectrophores is with 1% agarose gel. The forward primer-338F (5'- ACTCCTACGGGAGGCAGCAG-3') and reverse primer- 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the bacterial 16S rRNA gene's hypervariable region V3-V4.The PCR reactions were done in triplicate 20μL mixture containing 0.8μL of each primer (20 μM), 2 μL of 2.5 mM dNTPs, 10 ng of template DNA as well as 0.4 μL of FastPfu Polymerase. Following program were used to conduct the amplification which consist of early denaturation at 95°C for 3min, then 27 cycles, where 1 cycle is comprised of 95°C for 30s, 55°C for 30s of annealing and extension for 30s at 72°C as well as a final extension at 72°C for 10 min. PCR was completed by PCR thermocycler (GeneAmp 9700, ABI, USA). The resulted PCR products were extracted from a 2% agarose gel and then purified through the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). After that, using QuantiFluor™-ST (Promega, USA) those purified DNA were quantified.

Illumina MiSeq sequencing

In equimolar and paired-end sequenced (2 × 300) purified amplicons were pooled on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the typical protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Processing of sequencing data

The raw fast q files were demultiplexed, quality-filtered using Trimmomatic and then merged by FLASH meeting the following criteria: (i) The reads were shortened at any site receiving an average quality score above 20 over a 50 bp sliding window. (ii) Primers were exactly matched allowing 2 nucleotidemis matching, as well as reads containing ambiguous bases were removed. (iii) Sequences were merged by their overlap sequence whose overlap were longer than 10 bp. Operational taxonomic units (OTUs) using UPARSE (version 7.1 http://drive5.com/uparse/) were clustered with 97% similarity cutoff and by the help of UCHIME, chimeric sequences were recognized and detached. Each 16S rRNA sequence taxonomy was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) compared with the Silva (SSU128) 16S rRNA database with confidence threshold of 70%.

Diversity, PCoA analysis and cluster analysis

For specifying the diversity of microbiota in every sample, α-diversity index was calculated. Every index was equated among groups by the help of Kruskal-Wallis test (KW) in the SAS V.9.3. The richness of the most abundant 30 OTU was plotted for each sample in heatmap plot using the made4 package in R 3.1.1. Based on Bray Curtis distance using dist. shared Primary co-ordination analysis were performed and PCoA command sequentially in Mothur. With samples from the same patient connected, the PCoA co-ordination was re-plotted.

LEfSe analysis and non-redundancy

To calculate biomarkers among groups LefSe (linear discriminant analysis coupled with effect size measurements) analysis. When LefSe was analyzed between major microbiota clusters, to filter biomarkers a stricter one-against-one comparison was implemented. All these analyses have been done on website (www.i-sanger.com)

RESULTS

F comprised of 31 foreign healthy students with mean age of 24±6 year and C comprised of 11 healthy Chinese students with mean age of 25±4 year. F consisted of students coming from different countries which include Pakistan, India, Bangladesh, Zambia, Iraq, Saudi Arabia, Nigeria, Sudan, Sierra Lion, Tanzania, Somalia, Brundi and South Africa, while C consisted of Chinese students living in mainland China. A total of 1, 599, 407 sequences (short reads) were analyzed after passing the initial QT (Quality Control). The number of reads per individual ranges from 30,283 to 44,671 in F group and from 30,731 to 44,473 in C group; this difference was not significant. The average read length in F group was 435±3.40 (mean±SD) and in C group was 435±2.85 (mean±SD). The ability to successfully match the reads to published sequences was very high in both F (99.88%) and C (99.89%) groups.

The α diversity of microbiota

The general characteristics of alpha diversity are depicted in the Table 1. It's a numerical measure that reflects in a community diverse number of species are present. Diversity indices were used to deliver more evidence about composition of a community than merely species richness and also explain the number of different present species. They are not substitutable. Regardless of the strong associations between these diversity measures, there has been much discussions over which is more suitable in numerous contexts. The Shannon diversity index is a popular diversity index that refers to the diversity of species in a community. As we can see in the table 1, the mean value for Shannon index is larger in F group (3.502±0.520) than that of C group (3.283±0.318), with no statistical difference. Simpson's diversity index (*D*) calculates a diversity score for a community. The mean value of Simpson index is marginally lower for F group (0.081±0.046) as compared to C group (0.084±0.029), also with no statistical difference. The Mean for few other diversity indices was also plotted and are mentioned in the Table 1.

Table 1. Comparison of Richness and Diversity of Microbiota among Chinese and Foreign students

Estimator	C-Mean	C-Standard Deviation	F-Mean	F-Standard Deviation	P Value	Q Value
Sobs	183.64	58.255	231.19	63.82	0.06294	0.255
Shannon	3.283	0.318	3.502	0.520	0.1697	0.255
Simpson	0.084	0.029	0.081	0.046	0.548	0.548
Ace	220.22	78.671	259.22	70.93	0.1697	0.255
Chao	214.04	75.364	261.22	74.906	0.09144	0.255

C means: Chinese students
F means: Foreign students

F group includes more special OTUs

The overlap of OTU clusters between Chinese and Foreign samples was calculated, and the number of shared OTUs were demonstrated using the Venn Diagram (Fig. 1).720 is the total sum of OTUs between the 2 groups, from which the greatest number of OTUs were shared between them. 492 (68.33%) OTUs are present in the F group and 228 (31.66%) OTUs are in C group. The shared number of OTUs between the 2 groups is 420 (58.33%). The number of OTUs in the F group were higher which stats more diversity in this group.

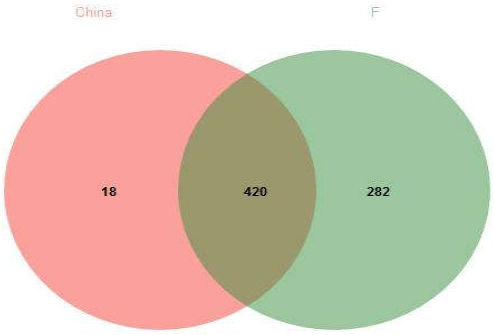

Figure 1. Venn Diagram showing shared OTUs between the 2 groups. A Venn diagram displaying the degree of unique and shared Operational Taxonomic Units (OTUs) between the two groups. Red circle represent Chinese students and Green circle represents Foreign students. The OTUs in the figure are indicated by numbers

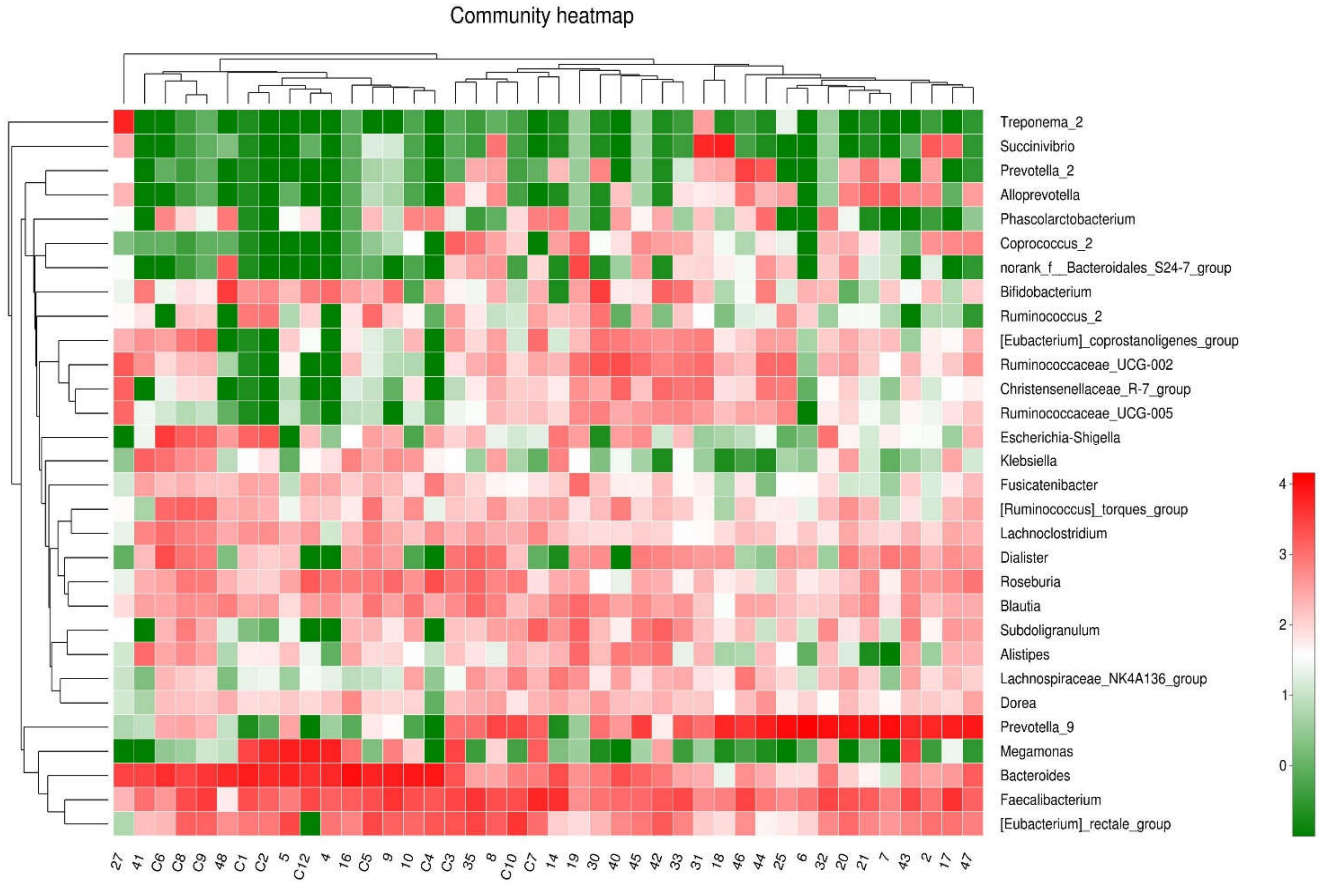

Figure 2. Community heatmap analysis of Gut Microbiota. A heatmap diagram visualizing the differentially abundant OTUs identified among the Chinese and Foreign students. Color bar and scale are provided in the figure (green color mean lower relative abundance while red color mean higher relative abundance)

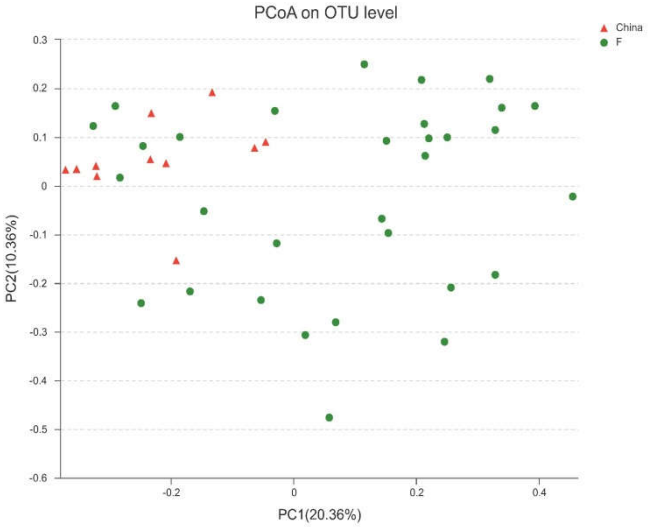

Figure 3. PCoA analysis on out level of the microbiota. Principal component analysis was identified in the two groups together, plotted according to their occurrence in Chinese (China) and Foreign (F) groups. PC_1 explained 20.36% of the variation observed, and PC_2 explained 10.36% of the variation. However, the samples form well separated clusters suggesting that the bacterial structures in both groups were not similar

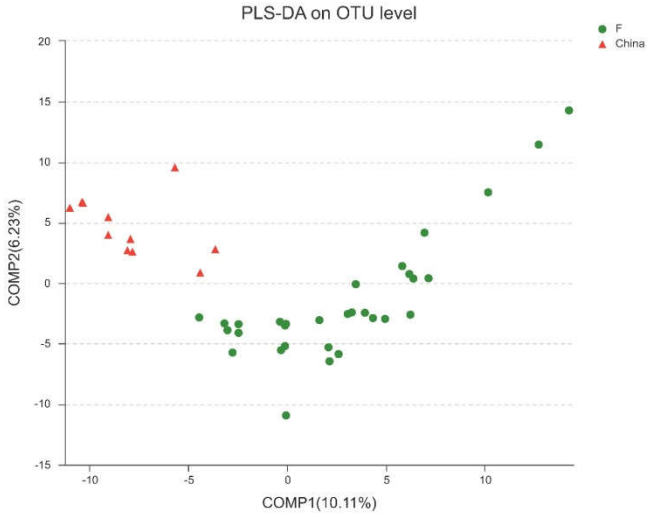

Figure 4. PLS-DA analysis of the microbiota. Partial Least Square Discriminate Analysis (PLS-DA) include variables with significant differences between the Chinese and Foreign samples. There are few inter-group similarities but overall there is significant diversity between the two groups

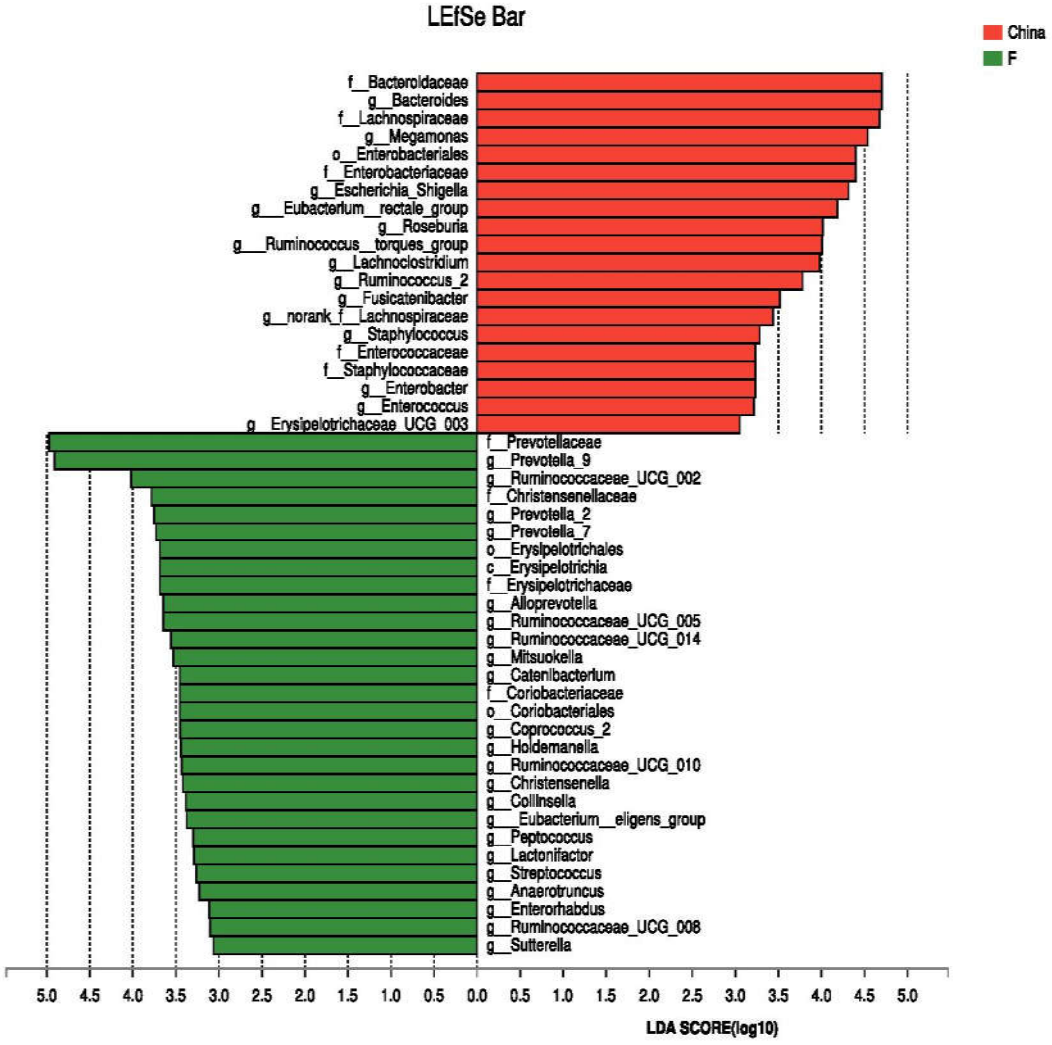

Figure 5. Comparison of microbial variations at the genus level, using the LEfSe. Taxa are arranged in descending order according to their LDA score. We identified the differently abundant genera with LEfSe (at LDA threshold of 3)

Community heatmap of gut microbiota

The OUT relative abundance as a heat map was shown in Fig.2.The result indicated that there was three enterotypes including Bacteroides, *Prevotella* and *Ruminococcaceae*. Higher proportion of Bacteroides was present in Chinese group and the concentration of *Prevotella* is higher in foreign group. *Ruminococcaceae* is the highest shared taxa between them with very marginal sharing of *Bacteroides* and *Prevotella*.

Different structure in the gut microbiota between C group and F group

Between healthy Chinese and foreign students very noteworthy differences were present in the total microbiota composition and are illustrated in the Fig. 3. PCoA (Principal Co-ordinate Analysis) were performed to check any gathering pattern between the fecal bacteria. There is no visual overlapping in any one of them. We further perform the analysis of similarities (ANOSIM). The results suggested that the structure of microbiota of F group was changed fromC group with no statistical difference (ANOSIM, R=0.1075, *P*=0.098). To determine whether it was possible to distinguish between the C and F group, we also carried out partial least square-discriminant analysis (PLS-DA) in Fig.4. The two groups were well separated as shown by the score plot based on the 2 components. These results indicate that their intestinal microbiota composition was significantly different from each other, but few of the samples were close to their individual partners of particular group.

LefSe analysis of the microbiota

Using LEfSe a managed evaluation was then made to statistically explain (at log LDA threshold of 3) the specific alterations in microbial structure among Chinese and Foreign students. This clarified that microbes from the genera Bacteroides and Lachnospiraceae was rich in the Chinese individuals and the genera Prevotella and Ruminococcoceae were more abundant in Foreign individuals (Fig. 5). Taxa are arranged in descending order according to their LDA score. We identified the differently abundant genera with LEfSe (at LDA threshold of 3).

DISCUSSION

Our research investigated the diversity of intestinal microbiota in healthy Chinese belonging to one province and healthy Foreigners coming from different countries via a highly innovative scheme. By the implement of extremely reproducible phylogenetic microarray analysis, very prominent differences were found among the healthy foreigners and Chinese students. The fecal microbiota in both groups of individuals was enriched with Firmicutes which were the most abundant phylum. Firmicutes include bacteria belonging to Clostridial clusters including Ruminococcus and *Erysipelotrichia*. All these bacteria ferment carbohydrates with the production of SCFAs (Flint *et al*. 2012; Russell *et al*. 2013). SCFAs are a source of energy to the colonic epithelium, influence metabolism in other parts of the body, especially the liver, and help in epithelial restitution and recovery from damage in the colon(Ramakrishna and Roediger 1990). Other members of the Firmicutes present in the Chinese group include Megamonas, *Lachnospiraceae*, *Lachnoclostridium, Staphylococcus* and *Eubacteriumrectale,* many of which are also beneficial to human health through metabolic and immune effects. The members present in the F group include *Christensenella, Mitsuokella, Catenibacterium, Coprococcus, Holdemanella, Peptococcus, Lactonifactor, Streptococcus* and *Anaerotruncus*. The principal bacterial groups among the Chinese and foreigners at the phylum-like level are found to be similar, with *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* being the principal phyla. *Firmicutes* and *Bacteroidetes*, from other studies investigations are found to be are the chief bacterial groups, which are responsible for 50-70% and 20-40% of the overall bacteria in adults, respectively (Ley *et al*.,, 2006; Frank *et al*., 2007; Yatsunenko *et al*., 2012; Andersson *et al*., 2008; Eckburg *et al*., 2005), these findings are also steady with them. Amazingly, the most profound bacterial group in both the Chinese and foreigners is found to be *Clostridium* cluster *XIVa*. They are gram negative bacteria. The major element of the *Firmicutes* phylum is the *Clostridium XIVa* and they have a large sum of significant butyrate-producing bacteria counting *Ruminococcus*, which is responsible for more than 12% of the overall microbiota in F group and around 5% in Chinese group. They are in control of providing the butyrate to the host, which is fatty acid of a short-chain and is thought to be a chief source of power for intestinal absorptive cells (Enterocytes). It performs few important functions, like supporting the mucosal physiology by increasing motility and controlling the immunity function of the intestinal epithelial cells and epithelial barrier as well (Thibault *et al*., 2010).

Bacteroidetes, which are abundant in many Western populations constituting much of the fecal microbiota, were comparatively low in Chinese group. The phylum Bacteroidetes harbor's many microbial genera, including Bacteroides and Prevotella. No doubt, there are huge number of differences among the Chinese and foreign students that can have an impact on the microbiota. A strong and effective explanation would be given by dietary differences (Wu *et al*. 2011). Our study had several limitations. Only taxonomic data were examined using amplification of 16S rRNA gene hypervariable regions V3-V4. Thus, interpretation of metabolic function in these two groups was not possible. Since only HVRs 3 and 4 were amplified, it was not always possible to assign taxa at the species level. The data pertained only to adult populations and this particular study did not evaluate differences at different age levels. In conclusion, this study provided interesting and previously unavailable data on gut microbial communities present in such diverse Foreign students. It would be interesting to determine to what extent these gut microbiota patterns occur in other universities students.

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